CELLULAR AND MOLECULAR STUDIES OF LARVAL OENOCYTE FUNCTION IN *DROSOPHILA*

by

Eugenio Gutierrez

Thesis submitted to the University of London,

for the degree of Doctor of Philosophy

December 2003

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my parents

ACKNOWLEDGEMENTS

First of all I would like to thank my supervisor Alex Gould. Alex took the risk of accepting me into his lab based mainly on a 90-minutes telephone interview. At my arrival he helped me to sort out my accommodation in the Cottages down the hill from the Institute and later on at Medresco House. This two things really made a difference in the type of living I have had here in London, making this not an awful stressful period but an extraordinary time. But the reason for which I am mostly grateful to Alex is for sharing with me his passion for science, always willing to discuss scientific issues that sometimes could go far beyond the scope of my project, ready to explain either the bases of several techniques or the underlying importance of certain discoveries, encouraging me to be both respectful and critic of other people work. He gave me great liberty regarding my project but reminded me quite often to keep focused and to think of testable hypothesis. In a few words I cannot think of a better Ph.D. supervisor.

I would also like to thank very much Patricia Serpente for being so supportive during these years. Patricia would always cheer me up in the lab and would help me in many senses. Her advice is wise and I learned a lot of things from her, both in terms of lab techniques but more importantly in human relationships. I am indebted to her.

Caterina Cenci is an extraordinary person and my relationship with her was as close as it could get as that with a sister. Caterina and I would often disagree but through that process I learned an awful lot from her. She would make me laugh in the most bizarre situations.

At my arrival to the lab, Bruno Bello and Véronique Brodu introduced me to the joys and pains of working with flies. Both of them are very knowledgeable and I thank them for their help. Phil Elstob, John Whitaker, Julia Pendred, Louise Cheng and Cedric Maurange provided a friendly atmosphere in the lab. To work with each one of them was great fun. Thanks to all members of the fly community at Mill Hill (Iris' lab, JP's lab and Gould's lab) for their advices and suggestions and specially for creating a great environment to work in.

I would also like to thanks Iain Robinson for maintaining an interest in my project and providing non-fly critical suggestions. Big thanks to Sergio Meza and Iris Estrada for being that inspiring and encouraging about a scientific career and to Iris for suggesting me to apply to Mill Hill.

Thanks to Lesley MacNeill, Loreta Wall and Frank Johnson for their help with some of the diagrams and models presented in this work.

Very special thanks to the friends I made during these years. Because of them, doing a degree abroad was not a story or loneliness but a vibrant time full of anecdotes and good memories. This people include Kathrin Schaefer, Rhian Kiely, Andrea Knowles, Liory Fern, Rina Sorathia, Randip Bains, Marta Fernández, Eleni Bazigou, Wendy Leung, Sara Ricardo, Sara de Silva, Sophia Tincey, Aminata Toure, Sophia and Patrick, Steve Renwick, Steen Ooi, Yunghan Au, Carl Shiu, Xavier Franch-Marro, Parvis Hakim and Paco Godefroy. Thanks to all of you guys.

I would also like to thank all the climbing lot for their friendship specially Richard Gibbons, Sandra Ruf, Matt Lock, Clive Lunny, Clare Lynch, Nicky Fenner, Ariel Achtman, Bimb, Sandra Kroenig and Bill Buass. I have enjoyed climbing sessions and trips more for you guys than for the climbs themselves.

This section would not have been complete without acknowledging Luis Antonio Menéndez and Miguel Angel López. Tony was key in encouraging me to undergo post-graduate studies and these three years are full of great stories and trips around Europe. Cheers mate. Mike is a terribly creative person whose conversations were inspiring and really funny. Here I would like to award my friends from the Peteretes group for the most silly and hilarious emails I received during this time, which, together with the Starcraft massacres, relaxed me from the stress of work. Thanks to many members of Clan 88 for keeping in touch and maintaining this bond alive.

I would like to thank all my family and friends back home for their encouragement and help. My sister Laura always became handy when I was in need to talk with someone that understood me, when I just wanted to have a laugh or to keep me informed of the latest gossips back home. The Cuevas Morales family hosted me on my first Christmas abroad making me feel at home and were always understanding of the things I went through living here. Them, together with my grandma, the Berumen Morales family, the Robles Morales family, the Arroyo family and the Morales Cabello family made me feel part of a community no matter the distance and each one of them made me feel home. Also thanks to Rossana Baños, Brenda García and Kenia Espinosa.

Finally, but not last, I would like to thank my parents Keny and Manolo, for their full support and encouragement throughout the length of my degree. They have always been there when I needed them and their advice have proved useful all along. Their lives will always be the best example for me. Thanks mom and dad, for encouraging me and helping me to make my dreams come true. I love you and this thesis is dedicated to you.

PREFACE

The research reported in this thesis was carried out in the Division of Mammalian Development at the MRC National Institute for Medical Research (Mill Hill, London), under the supervision of Dr. Alex Gould.

This thesis describes my own original work with the exception of Figure 3.8, A-C. These panels show preliminary work on oenocytes carried out by Dr. Alex Gould and were included to provide necessary background information.

This work was supported by funding from the Mexican National Council for Science and Technology (CONACyT) and the British Medical Research Council (MRC).

ABSTRACT

Lipid metabolism is regulated according to the nutritional status of the body and its energy demands. In vertebrates, the liver plays a central and multifunctional role in this process. Within hepatocytes, lipid metabolites of cytochrome P450-4 (Cyp4) enzymes serve to activate Peroxisome Proliferator-Activated Receptors (PPARs), leading to the induction of a myriad of genes involved in lipid uptake, processing and degradation. In principle, the insect Drosophila should provide a genetic model for studying these processes but several aspects of lipid metabolism, including the identity of the cell-types that fulfil the functions of the vertebrate liver are not yet clear.

The larval oenocytes of insects are large lipid-laden cells whose function has been a matter of considerable debate over the last hundred and fifty years. Three long-standing hypotheses are that oenocytes regulate tracheal respiration, hemolymph composition or aspects of moulting. To begin to test these hypotheses, I have used enhancer traps and a large database of expression patterns to identify 35 genes selectively expressed in oenocytes. 17 of these encode products highly related to vertebrate liver proteins playing roles in the uptake, modification and degradation of lipids. These include two lipophorin receptors, a Cyp4 protein and 6 peroxisomal enzymes catabolising fatty acids by ß-oxidation.

By using an optimised GAL4/UAS-reaper approach to ablate Drosophila oenocytes, I find that they are required for the completion of the moult from second-to-third larval instars. Arrested oenocyte-less larvae display duplicated exoskeletal elements, suppressed food intake and a premature wandering behaviour. These phenotypes are strikingly similar to those produced by mutations in the E75A variant of Ecdysone-induced protein 75B (Eip75B), an orthologue of vertebrate PPARs and one of the 35 oenocyte genes identified here. Together, these studies demonstrate that oenocytes possess much of the lipid-processing and regulatory circuitry of vertebrate hepatocytes and may use this machinery to trigger developmental transitions during larval life.

TABLE OF CONTENTS

CHAPTER FIVE: Discussion

LIST OF FIGURES AND TABLES

Chapter One

Chapter Two

Chapter Three

Chapter Four

Chapter Five

Appendices

ABBREVIATIONS

15d-PGJ2	15-deoxy-D12-14-PGJ2
17ß-HSD IV	17ß-hydroxysteroid dehydrogenase type IV
20-E	20-hydroxyecdysone
3ß-HSD	3ß-hydroxysteroid dehydrogenase/isomerase
8(S)-HETE	8(S)-hydroxyeicosatetraenoic acid
9-HODE	9-hydroxyoctadecadienoic acid
ABC	ATP-binding cassette
abdA	*abdominal A*
AcCoAS	Acyl Coenzyme A Synthase
Acrp30	Adipocyte complement related protein of 30 kDa
Adh	Alcohol dehydrogenase
Alas	*delta-aminolevulinate synthase gene*
AldhIII	Aldehyde dehydrogenase type III
amn	*amnesiac*
amon	*amontillado*
apo	Apolipoprotein
Atet	ABC transporter expressed in trachae
azPC	hexadecyl azelaoyl phosphatidylcholine
CAT	Carnitine acyltransferase
Cat	Catalase
CCAP	Crustacean CardioActive Peptide
COT	Carnitine-O-octanoyltransferase
COX	Cyclooxygenase
cpc	*Cryptocephal*
Cpr	Cytochrome P450 reductase
Cyp4	Cytochrome P450 family 4
Cyp4g1	Cytochrome P450 4G1
Cyps	Cytochrome P450 enzymes
dare	*defective in avoidance of repellents*
DBD	DNA-binding domain
DBP	Dorsal Bipolar Neurons

D-BP	D-Bifunctional Protein
dib	*Disembodied*
DILPs	*Drosophila* insulin-like peptides
dm	*diminutive*
E75A	Ecdysone-induced protein E75A
ecd	*ecdysoneless*
EcR	Ecdysone Receptor
EcRE	Ecdysteroid Response Elements
EGFR	Epidermal growth factor Receptor
Eip75B	*Ecdysone-induced protein 75B*
ELO	Long Chain-Fatty Acid Elongases
EPO	erythropoietin gene
ER	Endoplasmic Reticulum
ETH	Ecdysis-Triggering Hormone
FABP	Fatty Acid Binding Protein
FALDH	Fatty Aldehyde Dehydrogenase
FA	Fatty Acid
FB	Fat Body
FFA	Free Fatty Acid
GCS	Glycine Cleavege System subunit
GPCR	G Protein-Coupled Receptors
HDL	High-Density Lipoprotein
HETE	HydroxyEicosaTetraEnoic acid
HETrE	HydroxyEicosaTriEnoic acid
HIF-1α/ß	Hypoxia Inducible Factor 1 α/ß
HMG CoA	3-Hydroxy-3-MethylGlutaryl CoA reductase
Hnf4	*Hepatocyte nuclear factor 4*
hnt	*hindsight*
HPETEs	HydroPeroxyEicosaTetraEnoic acids
IPC	Insulin-Producing Cells
itpr	*IP-3 receptor*
JH	Juvenile Hormone

L1	First instar larvae
L2	Second Larval Instar
L3	Third Instar Larvae
LBD	Ligand-Binding Domain
LCFA	Long-Chain Fatty Acids
LDLR	Low-Density Lipoprotein Receptor
LDL	Low-Density Lipoprotein
LOX	Lipoxygenase
LPL	Lipoprotein Lipase
LpR	*Lipophorin Receptor*
LRP	LDL Receptor-Related Protein
LXR	Liver X Receptor
MCAD	*Medium Chain Acyl-Coa Dehydrogenase*
Met	Methoprene-tolerant
MFP-2	MultiFunctional Protein-2
m-NSC	median NeuroSecretory Cells
Msp-300	*Muscle-specific protein 300*
ngl	*new glue 1*
NHE	Na+ H+ exchanger
OEA	OleylEthanolAmide
ox-LDL	oxidized-LDL
P450s	Cytochrome P450 enzymes
PE	PhosphatidylEthanolamine
PGH2	ProstaGlandin H2
phm	*peptidylglycine a-hydroxylating monooxygenase*
pnt	*pointed*
PPAR	Peroxisome Proliferator-Activated Receptor
ppl	*pumpless*
PPRE	Peroxisome-Proliferator Response Element
PPs	Peroxisome Proliferator drugs
Prc	Pericardin
psq	*pipsqueak*

PTS	Peroxisomal Targeting Sequence
rho	*rhomboid*
RNAi	RNA interference
RXR	Retinoid X Receptor
sad	*shadow*
sal	*spalt*
SCAD	Short Chain Acyl-Coa Dehydrogenase
SCAP	SREBP cleavage-activating protein
SCP2	sterol carrier protein-2
SCPx	sterol carrier protein-X
shd	*shade*
sna	*snail*
Spi	Spitz
SRE	Sterol Regulatory Element
SREBP	Sterol Response Element Binding Protein
ßftz-f1	*ßfushi tarazu factor 1*
svp	*seven-up*
TG	TtriacylGlycerol
Tim17b2	*Translocase inner membrane 17*
TNFα	Tumor Necrosis Factor α
TZDs	ThiaZolideneDiones
usp	*ultraspiracle*
VLCFA	Very Long-Chain Fatty Acid
VLDL	Very Low-Density Lipoprotein

CHAPTER ONE

General Introduction

CHAPTER ONE: General Introduction

1.1 Scope of the Present Work

Organisms must adapt to changing environments. Because food sources are varied and not always plentiful, metazoans have evolved elaborate systems for maintaining internal homeostasis. Some of these systems regulate energy expenditure in line with the nutritional status of the organism and its particular stage of development. In all cases, the underlying bases for homeostatic control involves networks of specialised cell types communicating via humoral signals.

In the following introduction, I will describe the ways in which animals control energy expenditure, with an emphasis on lipid metabolism and hormonal controls. As *Drosophila melanogaster* will be used as the experimental system, aspects of these processes relating to this insect species will be highlighted. For those areas where there is not sufficient *Drosophila* information, descriptions in other species, mainly vertebrates, will be given. Finally, I will introduce the main subject of this thesis, the *Drosophila* larval oenocytes, explaining why previous work has drawn my attention towards their potential involvement in lipid metabolism and how I aim to provide further insights into their *in vivo* functions.

1.2 Lipid Homeostasis and Liver Function

Carbohydrates are the main short-term energy source and cells have elaborate metabolic pathways for maximising the energy that can be liberated rapidly from them. Lipids, on the other hand, are not as swiftly degraded by cells and represent the most effective way of storing energy per unit of mass, especially in the form of triglycerides (reviewed in (Berg, Tymoczko et al. 2001). Thus, while 1g of carbohydrate yields about 4kcal of energy, the same amount of fat produces 9kcal. On a normal diet, fatty acids (FAs) provide up to 40 % of the total energy consumed. But during fasting, they can become virtually the sole energy source.

Lipid metabolism is regulated such that there is a trade-off between usage of dietary lipids in energy production versus their long-term storage. Many factors can influence this, including nutrient availability, fluctuating energy demands of the organism and hormonal conditions.

1.2.1 Lipids are Transported as Lipoproteins

Because lipids contain hydrophobic moieties and are not generally water soluble, an elaborate system through which they can be transported from one tissue to another via the circulation has evolved (reviewed in (Lee, Olson et al. 2003). A series of huge macromolecular complexes termed lipoproteins, classified according to size, density and composition, play a critical role in this transport process. Each lipoprotein consists of a core of hydrophobic lipids, such as triacylglycerols (TGs) and cholesterol esters, covered with a layer of amphipathic phospholipids and polar proteins. The protein components of lipoproteins, called apoproteins, have multiple roles: they contribute to the overall structure of the lipoprotein, its solubilisation and, importantly, they also carry cell-targeting signals (reviewed in (Berg, Tymoczko et al. 2001). These signals allow different types of lipoprotein to be targeted to particular sets of tissues. Once at their appropriate destination, two mechanisms are used to release the various components of lipoproteins. One depends upon the action of extracellular lipoprotein lipases (LPLs) secreted by adipocytes, muscle cells and macrophages. LPL hydrolyses TGs into free fatty acids (FFAs) that are then able to penetrate into peripheral tissues. The second mechanism involves endocytosis of lipoproteins by target cells, a process mediated by a family of specialised lipoprotein receptors. Through specific apoprotein-receptor interactions, lipoproteins are internalised and delivered for degradation to lysosomes, where the apoprotein moieties are degraded into amino-acids and the lipid components are released into the cytosol (Willnow 1999). Whether the mechanism of LPL hydrolysis or receptor-mediated endocytosis is used depends on the particular type of lipoprotein (Table 1.1). Either way the lipids delivered by lipoproteins can act, not only as energy substrates but also as signalling molecules, such as ligands for various members of the nuclear hormone receptor family. In this latter role, lipids can produce long-term changes in cell metabolism (reviewed in (Lee, Olson et al. 2003).

1.2.2 The Liver is a Major Integration Point of Lipid Metabolic Pathways

The system transporting lipids between different body compartments can be divided into forward and reverse phases with respect to the central function of the liver (Figure 1.1). In forward transport, TG-rich lipoproteins are released by the intestine in the form of chylomicrons and by the liver in the form of very low-density lipoproteins (VLDLs). These lipoproteins deliver fatty acids to adipocytes for storage and to

Figure 1.1 The Liver as a Central Player in Lipid Metabolism

The liver is an integrative site for lipid homeostasis, where lipids are distributed to peripheral tissues for storage and energy consumption (Forward lipid transport) or captured from adipocytes and macrophages to be recycled or excreted (Reverse lipid metabolism). Lipid transit between these tissues occurs through several distinct types of macromolecular complexes termed lipoproteins, whose protein and lipid components vary (see Table 1.1). The Peroxisome Proliferator Activated Receptor (PPAR) family of transcription factors orchestrate the lipid-metabolic activities of all tissues (Modified from Willnow 1999 and Lee et al. 2003).

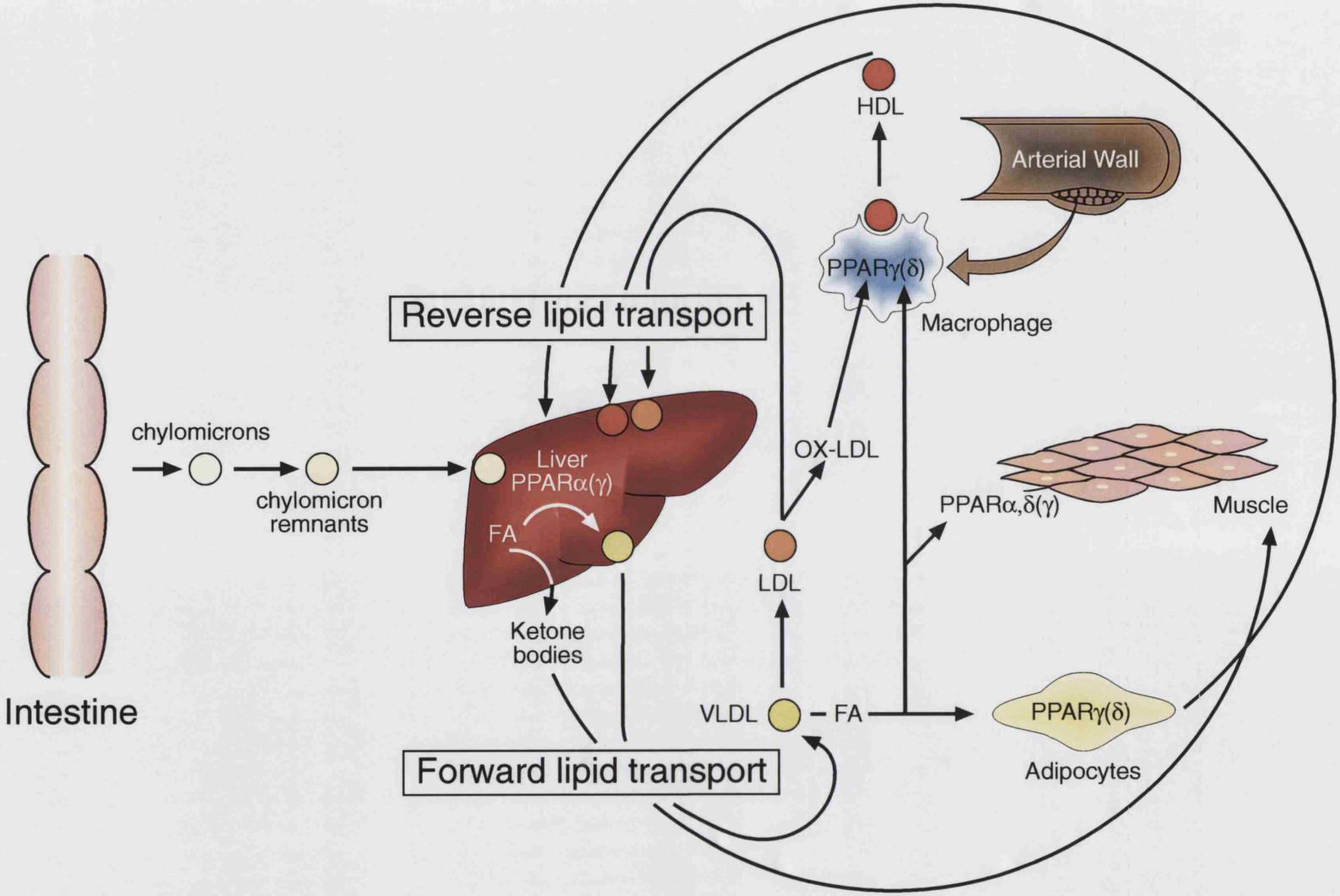

HDL
Arterial Wall
PPARγ(δ)
Macrophage
Reverse lipid transport
chylomicrons
chylomicron remnants
Liver PPARα(γ)
FA
Ketone bodies
OX-LDL
LDL
PPARα,δ(γ)
Muscle
Intestine
VLDL
FA
PPARγ(δ)
Adipocytes
Forward lipid transport

Table 1.1 Lipoproteins Transport Lipids Across Tissues

Lipoproteins differ in their lipidand protein components and the means by which these are released. See Figure 1.1 for explanation of Lipoprotein classes (Modified from Berg et al 2001).

Lipoproteins	Major core lipids	Apoproteins	Mechanism of lipid delivery
Chylomicron	Dietary triacylglycerols	B-48, C, E	Hydrolysis by lipoprotein lipase
Chylomicron remnant	Dietary cholesterol esters	B-48, E	Receptor-mediated endocytosis by liver
Very low density lipoprotein (VLDL)	Endogenous triacylglycerols	B-100, C, E	Hydrolysis by lipoprotein lipase
Intermediate-density lipoprotein (IDL)	Endogenous cholesterol esters	B-100, E	Receptor-mediated endocytosis by liver and conversion into LDL
Low density lipoprotein (LDL)	Endogenous cholesterol esters	B-100	Receptor-mediated endocytosis by liver and other tissues
High density lipoprotein (HDL)	Endogenous cholesterol esters	A	Transfer of cholesterol esters to IDL and LDL

muscle for energy consumption. In the circulation, LPLs release FAs from chylomicrons and VLDL, converting them into chylomicron remnants and low-density lipoproteins (LDLs), respectively. LDLs, which are rich in cholesterol esters, then deliver cholesterol to peripheral tissues for steroidogenesis and maintenance of cell membrane integrity. As the final step in their catabolism, chylomicron remnants and LDL enter the liver where they are taken up by hepatocytes via lipoprotein receptors (Willnow 1999; Lee, Olson et al. 2003). In reverse transport, FAs released from adipocytes, together with high-density lipoproteins (HDL) carrying excess cholesterol from peripheral cells such as macrophages, and residual LDLs, are transported to the liver for recycling or catabolizing into bile acids (reviewed in (Willnow 1999; Lee, Olson et al. 2003).

The vertebrate liver is a central site of metabolic integration, where FAs are mobilized and, depending on demand, either stored or used as an energy source (Figure 1.1, reviewed in (Lee, Olson et al. 2003). Under normal food intake conditions, the body uses both carbohydrates and lipids to obtain energy. During such times, the liver coordinates the synthesis of FFAs, the esterification of TGs, and their packaging into VLDL for export to adipocytes for storage and to muscle for energy consumption (reviewed in (Everett, Galli et al. 2000). During starvation, however, the carbohydrate supply quickly drops and energy is now obtained predominantly from lipids. In this state, stored FAs are released from adipocytes and captured by the hepatocytes of the liver. Here In the liver they are either re-esterified to TGs and assembled into VLDL or broken down through ß-oxidation to generate ultimately ketone bodies[1] (reviewed in (Lee, Olson et al. 2003). Hence, through these two processes of lipid anabolism and lipid catabolism, both of which are regulated by hormones and nutritional composition, hepatocytes are able to process a large amount of FAs without retaining them as TG droplets (reviewed in (Everett, Galli et al. 2000).

[1] Ketone bodies is a term generically used when referring to acetoacetate, D-3-hydroxybutyrate and acetone. The product of FA ß-oxidation is acetyl-CoA andunder normal nutrition, where there is a proper balance between lipid and carbohydrate degradation, it can enter the citric acid cycle by reacting with oxaloacetate to form citrate. However, during fasting, oxaloacetate is consumed to form glucose through the gluconeogenic pathway. In this state, acetyl-CoA produced by FA ß-oxidation is diverted into an alternative pathway that leads to the formation of ketone bodies. One advantage of ketone bodies, over the lipids from which they are produced, is that they are water-soluble and can diffuse from hepatocyte mitochondria into the circulation. When ketone bodies reach peripheral tissues, they are used as fuel for mitochondrial respiration. Even though the major site of ketone body formation is the liver, it cannot utilise them itself as it lacks the particular CoA transferase catalysing their activation (reviewed by Berg, J., J. Tymoczko and L. Stryer (2001). Biochemistry. New York, Freeman and Co.

1.2.3 Regulation Of Lipid Synthesis

One of the best understood lipid homeostatic mechanisms is a feedback system in mammals in which both FAs and cholesterol regulate their own synthesis (reviewed in (Nohturfft and Losick 2002). In this system, several crucial genes required for lipid synthesis are activated by sterol-response element binding proteins (SREBPs), transcription factors that bind to a short DNA sequence termed the *sterol regulatory element (SRE)*. In conditions where cholesterol and FAs are abundant in cell membranes, SREBPs remain complexed in the endoplasmic reticulum (ER) with another protein, SREBP cleavage-activating protein (SCAP). Upon depletion of cholesterol or FAs from cell membranes, SREBP-SCAP complexes translocate to the Golgi apparatus, where SREBPs are cleaved by two proteases. This cleavage allows membrane release and translocation of a transcriptionally-active fragment from the amino-terminus of SREBP into the nucleus where it can activate the transcription of genes involved in lipid synthesis. The exact details of how lipids mediate SREBP-SCAP translocation are not fully understood but it has been suggested that they bind directly to SCAP or to an adaptor protein. Alternatively, a reduction in cholesterol or FAs may modify the physical properties of the ER membranes thus activating the SREBP-SCAP complexes (Dobrosotskaya, Seegmiller et al. 2002; Nohturfft and Losick 2002). Importantly, insects are unable to synthesise cholesterol and therefore have to obtain it from their diet (reviewed by (Grieneisen 1994). Accordingly, *Drosophila* SREBP does not respond to sterol levels, but instead its proteolytic processing is inhibited by the unsaturated FA palmitate (Nohturfft and Losick 2002; Seegmiller, Dobrosotskaya et al. 2002). More specifically, it has been suggested that palmitate exerts this action specifically via its metabolite, phosphatidylethanolamine (PE), the major phospholipid in *Drosophila*. As in vertebrates, *Drosophila* SREBP activation leads to the expression of genes involved in FA and membrane lipid synthesis (reviewed in (Nohturfft and Losick 2002).

In vertebrates, control of lipid synthesis is known to be regulated at other levels too. For example, 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA), an enzyme involved in the committed step of cholesterol biosynthesis, is not only regulated at the transcriptional level by SREBPs but also at the post-transcriptional level by modulation of its degradation rate and phosphorylation state (reviewed in (Berg, Tymoczko et al. 2001). Similarly, acetyl CoA carboxylase, the enzyme

catalysing the committed step in FA synthesis is controlled at different levels. Its activity is modulated by phosphorylation, regulated by three hormonal signals, glucagon, epinephrine (adrenalin) and insulin. Acetyl CoA carboxylase activity is also modulated by the intracellular factors citrate, palmitoyl CoA and AMP, either by phosphorylation, as in the case of AMP, or by allosteric modification, as for citrate and palmitoyl CoA. Under well-fed conditions, insulin and high levels of citrate stimulate lipid synthesis but during starvation, epinephrine, glucagons and high levels of AMP inhibit it. When there is an excess of FAs, palmitoyl CoA also inhibits this process (reviewed in (Berg, Tymoczko et al. 2001).

In conclusion, several different mechanisms regulating lipid synthesis have been identified and well studied in mammals but similar work in *Drosophila* is only just beginning. Lipid degradation is also tightly controlled and in the following sections I will describe the subcellular machinery involved in lipid degradation (Section 1.3), and then explain how lipid degradation is coordinated with lipid synthesis and storage by the action of specific nuclear receptors (Section 1.4) and their potential ligands (Section 1.5).

1.3 Roles of Peroxisomes in Lipid Degradation

Peroxisomes are ubiquitous cell organelles that range from hundreds of nanometres to several micrometres in diameter and play a major role in lipid metabolism. Their name derives from the early observation that these organelles were capable of producing H_2O_2 as a by-product of the lipid degradation reactions they perform. Considerable interest in peroxisomes and their functions has arisen from the analysis of genetic diseases in which these organelles are absent or not fully functional, such as in Zellweger syndrome. This is a human pathology caused by mutations in any one of several genes involved in peroxisome biogenesis (Fujiki 1997). Zellweger syndrome patients display a range of defects, including craniofacial, neurological, ocular, hepatological and skeletal abnormalities. Studies of such diseases have proved useful for revealing that peroxisomes are involved in ß-oxidation of FAs, in α-oxidation of 3-methyl branched FAs and in biosynthesis of etherphospholipids (reviewed in (Wanders 2000).

1.3.1 Peroxisome Biogenesis

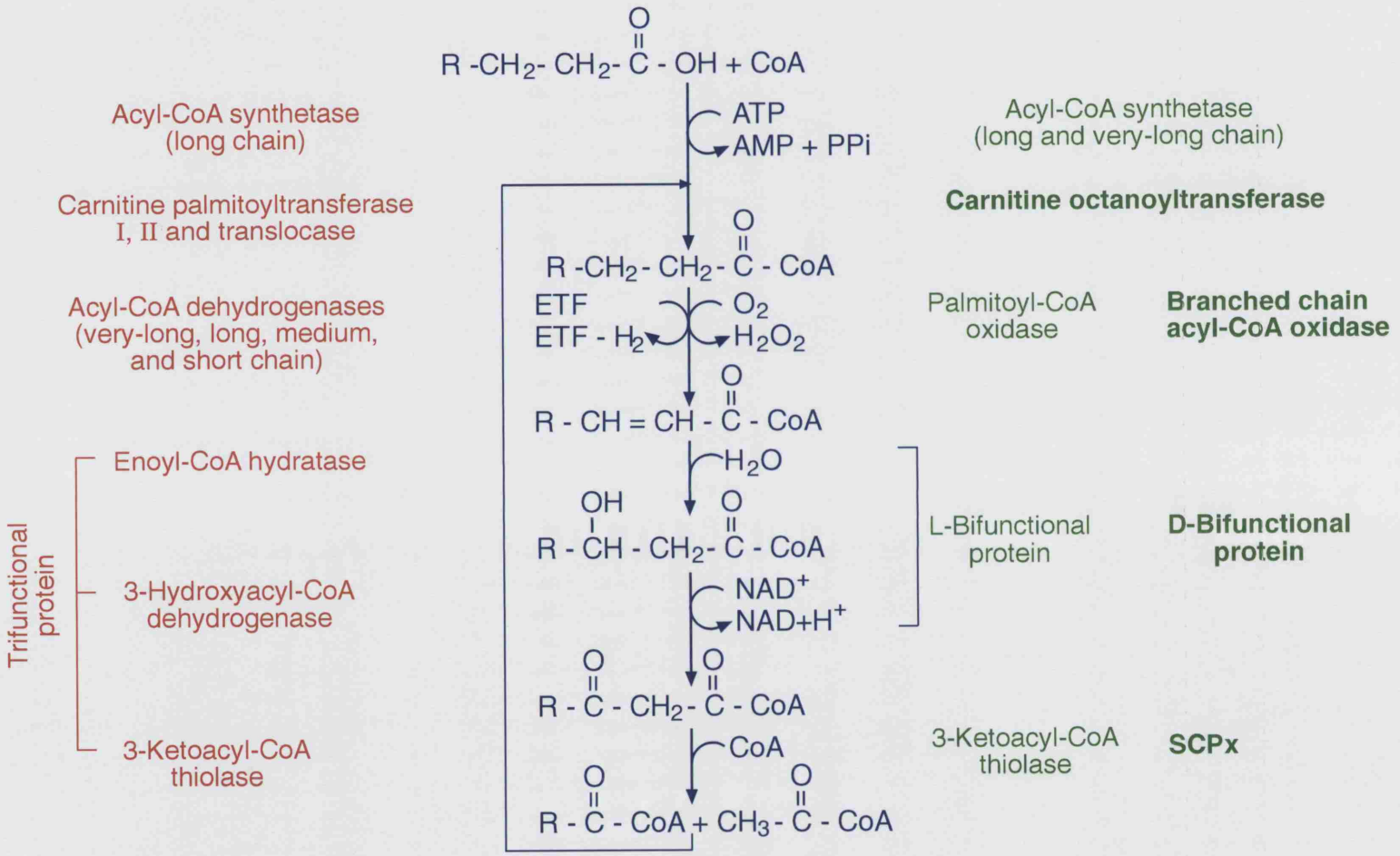
The biogenesis of individual peroxisomes does not appear to occur *de novo* but involves the import of proteins and other components into pre-existing organelles. These organelles then divide and form daughter peroxisomes that segregate during cytokinesis (reviewed in (Lazarow 2003). The molecular mechanism by which proteins are specifically incorporated into peroxisomes is not yet fully elucidated. However, it is known that several soluble proteins of the peroxisomal matrix are targeted by means of peroxisomal targeting sequences (PTS). Two of these have been well characterized: PTS1, a carboxy-terminal tripeptide (SKL or conservative variants, (Kal, Hettema et al. 2000; Emanuelsson, Elofsson et al. 2003) and references therein) and PTS2, an amino-terminal bipartite signal (consensus [RK][LVI]XXXXX[HQ][LA], (Emanuelsson, Elofsson et al. 2003). The targeting of proteins to the membrane of peroxisomes occurs by a different mechanism than that used for importing soluble matrix components. Two peroxisomal membrane proteins (Pex3p and Pex16p) are thought to be involved in the insertion of other new peroxisomal proteins into the membrane (reviewed in (Lazarow 2003). Pex19 a cytosolic peroxisomal protein is thought to assist in transporting newly synthesized peroxisomal membrane proteins from their site of synthesis to peroxisomes. (Lazarow 2003)

1.3.2 Fatty Acid ß-Oxidation by Peroxisomes and Mitochondia

The ß-oxidation pathway is the major route for lipid degradation. FA chains are shortened by the removal of two carbon atoms at a time, in the form of acetyl-CoA, in a spiral-type reaction. Both peroxisomes and mitochondria have the ability to ß-oxidise FAs. However, the different sets of enzymes present in each organelle, couple this process to different final outcomes, tailored for different functions (Figure 1.2). Thus, mitochondrial FA ß-oxidation is used for providing energy in a highly efficient manner, via the complete degradation of lipids into their C2-units that can then enter the citric acid cycle. Short, medium, and long straight-chain FAs are exclusively (short FAs) or mainly oxidised by mitochondria. Other compounds oxidised by mitochondria are medium-chain dicarboxylic FAs, medium-chain 2-methyl-branched FAs, and some xenobiotics with short carboxyl chains. On the other hand, the peroxisomal system is not as efficient in yielding energy from lipid oxidation. Instead, the limited number of rounds of FA ß-oxidation carried out within peroxisomes provides a chain-shortening mechanism that produces better substrates for subsequent

Figure 1.2 Fatty Acid ß-Oxidation by Mitochondria and Peroxisomes

Both organelles carry out the same biochemical reactions involved in FA catabolism, although a different set of enzymes is used in each case. Peroxisomal degradation is specialized for VLCFAs and LCFAs and bifurcates into two pathways, depending whether the substrate is a straight chain or a branched chain FA.Enzymes in bold represent the products of genes selectively expressed in oenocytes (see Sections 4.3.3 and 4.3.4, modified from Hashimoto 2000).


Mitochondria
Peroxisomes
R -CH2- CH2- C - OH + CoA
ATP
AMP + PPi
Acyl-CoA synthetase (long chain)
Acyl-CoA synthetase (long and very-long chain)
Carnitine palmitoyltransferase I, II and translocase
Carnitine octanoyltransferase
R -CH2- CH2- C - CoA
Acyl-CoA dehydrogenases (very-long, long, medium, and short chain)
ETF
ETF - H2
O2
H2O2
Palmitoyl-CoA oxidase
Branched chain acyl-CoA oxidase
R - CH = CH - C - CoA
H2O
Trifunctional protein
Enoyl-CoA hydratase
OH
R - CH - CH2- C - CoA
L-Bifunctional protein
D-Bifunctional protein
3-Hydroxyacyl-CoA dehydrogenase
NAD+
NAD+H+
R - C - CH2- C - CoA
CoA
3-Ketoacyl-CoA thiolase
SCPx
3-Ketoacyl-CoA thiolase
R - C - CoA + CH3 - C - CoA

ß-oxidation in mitochondria and also more suitable molecules for excretion. Peroxisomal substrates include very long-chain fatty acids (VLCFAs, such as prostaglandins, thromboxane and leukotrienes), long-chain fatty acids (LCFAs, both saturated and polyunsaturated), branched FAs (such as pristanic acid derived from dietary phytol), bile acid intermediates (di- and trihydroxycoprostanic acids), and the side-chains of xenobiotics that can not be catabolized by mitochondria. Once FAs have been shortened within peroxisomes, they can then either enter into mitochondria for further degradation (VLCFAs, LCFAs and 2-methyl branched FAs) or be excreted via the bile or urine (reviewed in (Hashimoto 2000; Mannaerts, Van Veldhoven et al. 2000). Importantly, unlike mitochondrial activity, peroxisomal activity is believed to be controlled solely at the transcriptional level.

In mitochondria, FAs are transported into the matrix by the carnitine acyltransferase (CAT)/acylcarnitine translocase system. In contrast, it has been suggested that passage of FAs across the peroxisomal membrane is mediated by various membrane transporters that possess an ATP-binding cassette (ABC transporters). Four of these have been identified (Shani, Jimenez-Sanchez et al. 1997), each constituting a half-transporter that, after homo- or heterodimerisation with another ABC family member, may form a complete and functional carrier (Hettema, van Roermund et al. 1996; Dean, Hamon et al. 2001; Dean, Rzhetsky et al. 2001).

The purification and characterization of enzymes of the peroxisomal ß-oxidation system was made possible by the initial discovery that isolated rat-liver peroxisomes are capable of FA oxidation (Lazarow and De Duve 1976). In both mitochondria and peroxisomes, FA oxidation involves the same chemical modifications of FA moieties. However, within each organelle these are carried out by different sets of enzymes (Figure 1.2). Once the FAs have been transported inside peroxisomes or mitochondria, they are activated by organelle-specific synthases, which covalently link them to Coenzyme A. Subsequently, they undergo successive rounds of oxidation, each round consisting of a series of four reactions. In mitochondria these steps are performed by the sequential action of an acyl-CoA dehydrogenase and a single enzyme catalysing three consecutive steps, termed trifunctional protein. The peroxisomal set of oxidation reactions can be divided into two pathways. One of them catalyses the ß-oxidation of straight-chain substrates and is inducible by peroxisome proliferator drugs (PPs). The second pathway acts on branched-chain acids, such as pristanic acid or bile-acid intermediates, and also on

straight LCFA and VLCFA, and is not induced by PPs. Both peroxisomal pathways degrade their substrates by the sequential action of an acyl-CoA oxidase (palmitoyl-CoA oxidase or branched chain acyl-CoA oxidases such as pristanoyl-CoA oxidase), a so-called bifunctional protein (L- or D-bifunctional protein) and a thiolase (3-ketoacyl-CoA or SCPx, (Hashimoto 2000).

In mitochondria the dehydrogenase reaction is indirectly coupled to oxygen via the electron transport system. In contrast, in peroxisomes, the first step of the ß-oxidation cycle is directly coupled to oxygen and generates hydrogen peroxide. This highly reactive and potentially damaging product is inactivated by a peroxisomal enzyme called catalase. This is unique to peroxisomes and thus provides a useful marker for this organelle.

Both mitochondria and peroxisomes contain carnitine acyltransferases. However, while in mitochondria they are involved in the penetration of fatty acyl-CoAs into the matrix, peroxisomal carnitine transferases are believed to convert medium-chain acyl-CoAs produced by peroxisomal ß-oxidation (no smaller than octanyl-CoA) into acylcarnitines that can then enter mitochondria for further degradation (reviewed in (Mannaerts, Van Veldhoven et al. 2000; Berg, Tymoczko et al. 2001).

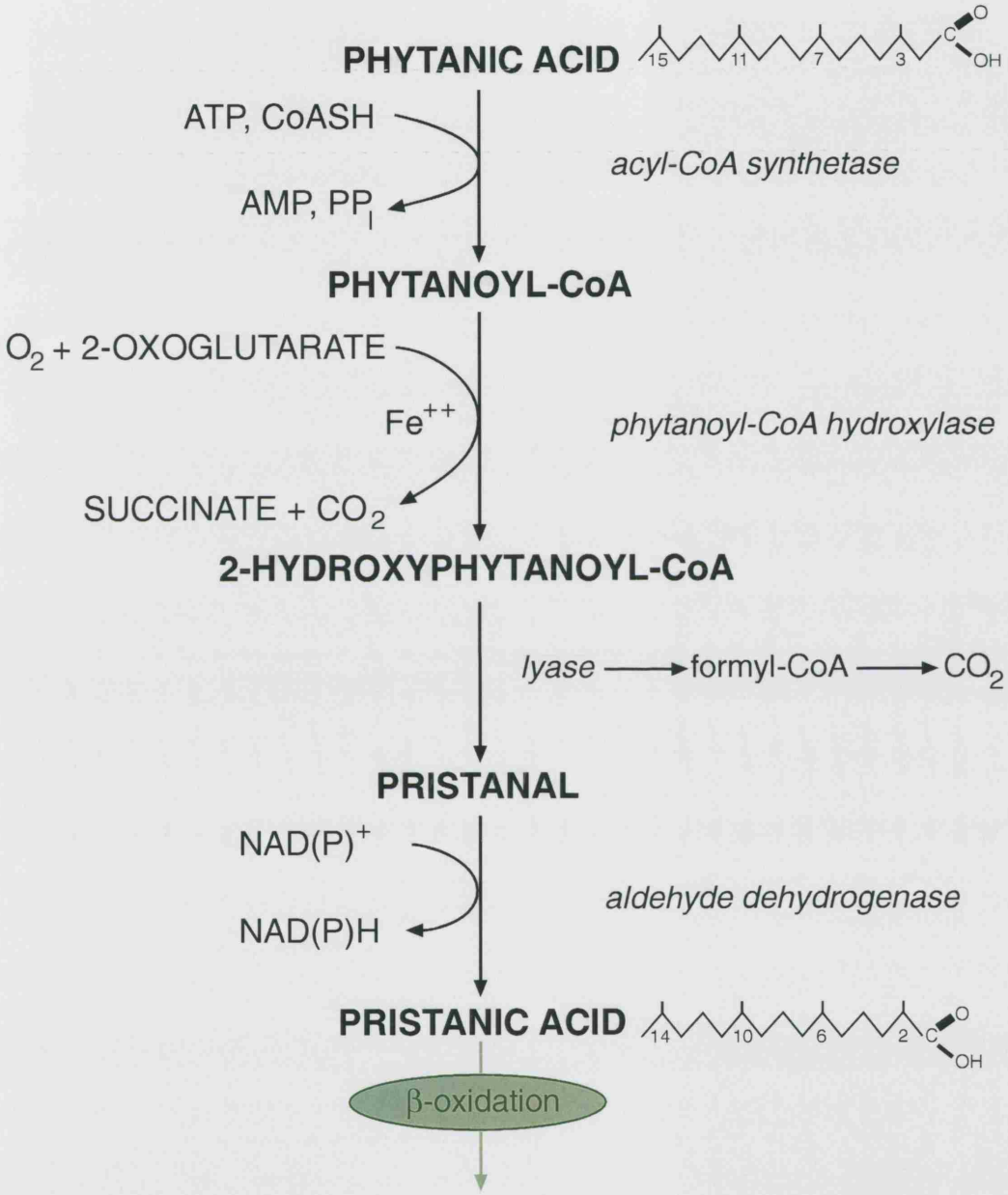
In summary, degradation of FAs by ß-oxidation involves metabolic coupling between peroxisomes and mitochondria. Both organelles perform the same types of oxidative reactions, with mitochondria specialised in maximizing the amount of energy obtained from each FA, while peroxisomes mainly come into play under conditions of FA excess and have a preference to somewhat longer-chain FAs.

1.3.3 Fatty Acid α-Oxidation

FA α-oxidation is an auxiliary pathway that must be used by 3-methly branched FAs, such as phytanic acid, before they can be degraded into suitable substrates for the ß-oxidation pathway (Figure 1.3). Such branched FAs are first degraded by α-oxidation (Wanders 2000) to produce 2-methyl branched compounds, such as pristanic acid, which can then enter the ß-oxidation pathway (reviewed in (Wanders 2000). In the rat, the entire α-oxidation pathway appears to be peroxisomal (Croes, Casteels et al. 1995). In humans, however, although the early steps (activation by coupling to Coenzyme A and hydroxylation) are peroxisomal, the intermediate step (cleavage)

Figure 1.3 The Fatty Acid α-Oxidation Pathway

Diagram showing the α-oxidation of phytanic acid, a 3-methyl branched FA. This pathway removes one carbon atom from the carboxy-terminus of 3-methly branched FAs, producing 2-methyl branched products which can then enter the ß-oxidation pathway (Modified from Petryk et al. 2003).


PHYTANIC ACID
15
11
7
3
O
OH
ATP, CoASH
AMP, PPi
acyl-CoA synthetase
PHYTANOYL-CoA
O2 + 2-OXOGLUTARATE
Fe++
SUCCINATE + CO2
phytanoyl-CoA hydroxylase
2-HYDROXYPHYTANOYL-CoA
lyase
formyl-CoA
CO2
PRISTANAL
NAD(P)+
NAD(P)H
aldehyde dehydrogenase
PRISTANIC ACID
14
10
6
2
β-oxidation

seems to occur in both peroxisomes and the ER, while the location of last step (dehydrogenation) remains unclear (reviewed in (Mannaerts, Van Veldhoven et al. 2000).

In conclusion, peroxisomes are cell-ubiquitous organelles specialised for lipid degradation. The multiple peroxisomal defects associated with Zellweger syndrome have been used to dissect out the various steps in this process and their function in human tissues. In particular, lipid degradation by the peroxisomes and mitochondria of hepatocytes is regulated to maintain overall lipid homeostasis in the face of changing demands by peripheral tissues.

1.4 Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome Proliferator-Activated Receptors (PPARs) are nuclear hormone receptors that play a central role in lipid regulation (Figure 1.1). Early studies demonstrated that a diverse set of compounds was able to induce the proliferation of peroxisomes in rat hepatocytes (reviewed in (Everett, Galli et al. 2000). Later it was shown that these compounds work by activating PPARs. There are three PPAR subtypes, PPARα, PPARß/δ and PPARγ, which differ primarily in their ligand-binding domains and domains of expression. PPARs are thought to sense lipid components of lipoproteins and to use this information to co-ordinately regulate lipid-metabolic target genes in several different target tissues (reviewed in (Lee, Olson et al. 2003).

PPARs are of considerable clinical importance as they are the molecular targets of hypolipidemic and insulin-sensitising drugs. For example, PPARα is the molecular target of fibrates, a lipid-lowering class of drugs; and synthetic activators of PPARγ, such as thiazolidenediones (TZDs), improve insulin sensitivity in humans with type II diabetes (Sood, Colleran et al. 2000). Several FA-derived molecules have been implicated as endogenous ligands for PPARs (Forman, Tontonoz et al. 1995; Kliewer, Lenhard et al. 1995; Forman, Chen et al. 1997; Kliewer, Sundseth et al. 1997; Krey, Braissant et al. 1997; Kliewer, Lehmann et al. 1999; Lim and Dey 2002; Nosjean and Boutin 2002; Fu, Gaetani et al. 2003). However, the identification of all of the endogenous PPAR ligands is far from complete and it is generally believed that many, as yet unknown, lipid ligands remain to be discovered.

PPARs function as heterodimers with retinoid X receptors (RXRs). Such heterodimers bind to a consensus motif known as the peroxisome-proliferator response element, (PPRE). This usually consists of two direct repeats of the hexad [A/G]GGTCA with a spacer of one nucleotide (DR-1) or two nucleotides (DR-2, reviewed in (Everett, Galli et al. 2000). At least one additional receptor, liver X receptor (LXR) has been shown to form heterodimers with PPARα, decreasing its activity (reviewed in (Everett, Galli et al. 2000). Heterodimers of other transcription factors present in liver, such as Hnf4, COUP-TF and ARP-1 with RXR can also bind DR-1 elements, raising the possibility that interactions between all of these receptors may allow the fine-tuning of PPRE-containing gene expression.

1.4.1 PPAR Isoforms and their Potential Endogenous Ligands

PPARα

PPARα potentiates FA catabolism in metabolically active tissues such as liver, heart, muscle and kidney. Earlier studies demonstrated that, in the liver, PPARα directly regulates many FA-metabolic genes reviewed in (Simpson 1997; Chawla, Repa et al. 2001; Okita and Okita 2001). The proteins encoded by these genes are involved in FA uptake and export of FAs in VLDL (such as FA binding protein (FATB) and various apolipoproteins), microsomal FA ω-oxidation (such as cytochrome P450 Cyp4, see Section 1.5.5), peroxisomal and mitochondrial FA ß-oxidation (such as peroxisomal ABC transporters, carnitine octanoyltransferase, acyl-CoA oxidase, catalase, SCPx, peroxisomal thiolase, medium-chain acyl-CoA dehydrogenase, carnitine acyl-transferase I , (Sharma, Lake et al. 1988; Sharma, Lake et al. 1988; Dreyer, Keller et al. 1993; Choi, Oh et al. 1995; Peters, Hennuyer et al. 1997; Cui, Kawashima et al. 2001; Fourcade, Savary et al. 2001; Girnun, Domann et al. 2002; Lopez, Irby et al. 2003). Analysis of PPARα knockout-mice confirmed that this isoform is essential for the upregulation of these genes in a fasting state (Kersten, Seydoux et al. 1999; Leone, Weinheimer et al. 1999).

Recently, oleylethanolamide (OEA) has been identified as an endogenous high-affinity activator of PPARα, being able to regulate expression of PPARα target genes in liver and small intestine, increasing lipid catabolism, at the same time than inducing satiety (Fu, Gaetani et al. 2003). Other ligands of PPARα with lower affinity

than OEA include leucotriene LTB4 and 8(S)-hydroxyeicosatetraenoic acid (8(S)-HETE, (Funk 2001).

PPARß

PPARß, also known as PPAR∂, is expressed ubiquitously and its functions are less well defined than those of the other PPARs. However, it has been implicated downstream of VLDL signalling to the macrophage (Chawla, Lee et al. 2003). In these cells, PPARß/∂ activation is mediated by the binding of components of VLDL, released by LPL (Londos, Brasaemle et al. 1999; Chawla, Lee et al. 2003), reviewed in (Ginsberg 2002; Lee, Olson et al. 2003). One potential ligand for PPARß is prostacyclin PGI2, one of the major prostaglandins, that plays other well known roles as an anticoagulator for platelets and as a strong vasodilator (reviewed in (Lim and Dey 2002)

PPARγ

PPARγ is highly enriched in adipocytes and foam cells (lipid-laden macrophages that have been associated with early stages of atherosclerotic plaques). It is involved in adipocyte differentiation, lipid storage and glucose homeostasis (Barak, Nelson et al. 1999; Kubota, Terauchi et al. 1999; Rosen, Sarraf et al. 1999), reviewed in (Chawla, Repa et al. 2001; Lee, Olson et al. 2003). It plays an important role in the balance between lipid influx from adipocytes and efflux into the blood stream (reviewed in (Lee, Olson et al. 2003). FFAs released by adipocytes normally enter the circulation to be taken up by the liver. However, upon PPARγ activation in adipocytes, there is an increase in FFA and glucose uptake and also in lipid storage. A key event in this process is the upregulation of the PPARγ target, the *LDL Receptor-related protein* gene *(LRP)*, encoding a membrane receptor involved in lipoprotein uptake by adipocytes (Gauthier, Vassiliou et al. 2003). In addition, PPARγ activation modulates the secretion of adipocyte-derived signalling molecules such as the appetite modulator leptin, tumor necrosis factor α (TNFα) and adipocyte-complement-related protein of 30 kDa (Acrp30, reviewed in (Lee, Olson et al. 2003). The most well characterised PPARγ ligands are 18-carbon and 20-carbon long (eicosanoids) FA-derived compounds. The best described of these compounds are the products of the enzyme lipoxygenase (LOX), such as 9-hydroxyoctadecadienoic acid (9-HODE), 13-HODE

and 15-HETE, and the cyclooxygenase (COX) products, such as prostaglandin 15-deoxy-Δ12-14-PGJ2 (15d-PGJ2). These compounds bind PPARγ with low affinities in the micromolar range. Recently, however, a high-affinity PPARγ ligand, an oxidised phospholipid called hexadecyl azelaoyl phosphatidylcholine (azPC), has been identified (reviewed in (Nosjean and Boutin 2002). Interestingly, several ligands for PPARγ such as 9-HODE, 13-HODE and azPC are components of oxidized-LDL (ox-LDL) and therefore provide means to activate transcription in foam cells upon LDL uptake (Nagy, Tontonoz et al. 1998; Tontonoz, Nagy et al. 1998), reviewed in (Nosjean and Boutin 2002).

1.5 VLCFA-Derived Signalling Molecules and Intracellular Messengers

In the previous sections I illustrated the central role of one particular cell-type, the hepatocyte, in lipid metabolism. Then, at the subcellular level, the functions of mitochondria and peroxisomes in lipid degradation were highlighted. Finally, at the molecular level, the role of PPARs in activating batteries of lipid metabolic genes was described. As VLCFAs, such as the eicosanoids, have been proposed as PPAR ligands, in the following section I will briefly outline their biological functions and then describe their biosynthesis.

1.5.1 VLCFA synthesis

FA synthase, the enzyme that generates FAs by the successive addition of two-carbon atoms units, has a limited range of elongation, its major final product being palmitate, a saturated FA with a backbone of 16 carbons and no carbon-carbon double bond (C16:0, (Berg, Tymoczko et al. 2001). However, animals use a vast array of longer, usually unsaturated FAs and their derivatives as membrane components, extracellular signals and intracellular messengers (Berg, Tymoczko et al. 2001). To obtain FAs longer than 16 carbon atoms, animals have to synthesise them by an alternative to FA synthase or they must obtain them from the diet.

Endogenous generation of VLCFAs is achieved by two types of accessory enzymes that catalyse either the elongation or desaturation of FAs. Both classes of enzyme localise to the cytosolic face of the ER membrane (reviewed in (Berg, Tymoczko et al. 2001). FA elongation is achieved by sequential addition of two-carbon atom units to the carboxyl end of both saturated and unsaturated fatty acyl

CoA substrates. Enzymes containing such an activity are referred to as elongases. Different species contain several genes encoding elongases each preferring particular FAs as their substrates. The *C. elegans* genome contains 8 elongase genes, all of which have been analysed by loss of function approaches and some of which have been biochemically characterised (Kniazeva, Sieber et al. 2003). In *Drosophila*, there are at least 21 predicted elongases, none of which have yet been genetically or biochemically characterised. Desaturation of FAs is mediated by a set of three membrane-bound enzymes: NADH-cytochrome reductase, cytochrome b5 and a desaturase (reviewed in (Berg, Tymoczko et al. 2001). Through the combined actions of different elongation and desaturation systems, a variety of unsaturated VLCFAs can be formed.

Despite the presence of elongation and desaturation systems, not all VLCFAs used by vertebrates can be produced endogenously. For example, mammals lack the enzymes required for introducing double bonds at carbon atoms beyond the C-9 position in the FA chain. Therefore, essential FAs such as linoleate (18:2 cis-Δ9, Δ12) and linolenate (18:3 cis-Δ9, Δ12, Δ15) must be obtained from the diet (reviewed in (Berg, Tymoczko et al. 2001). Some of these VLCFAs undergo further biochemical modifications to become important signalling molecules, as the ones that will now be described.

1.5.2 The Eicosanoids

Eicosanoids is a collective name given to the group of VLCFA-derived molecules that contain a 20-carbon backbone. This group comprises the prostaglandins, prostacyclins, thromboxanes, leukotrienes, hydroxy-eicosatetraenoic acids (HETEs), hydroxyeicosatrienoic acids (HETrEs) and hydroperoxyeicosatetraenoic acids (HPETEs, (Voet and Voet 1995). Eicosanoids are short-range hormones that have been implicated in a wide range of biological processes in practically all cell-types (reviewed in (Voet and Voet 1995; Funk 2001; Nebert and Russell 2002). Examples of these processes include inflammatory responses, pain responses, regulation of blood pressure, reproductive functions, regulation of sleep/wake cycles and bone resorption.

Eicosanoids are very potent and can act at extremely low concentrations in the nanomolar and sometimes picomolar range, but are very unstable and have half lines of a few minutes or less (Voet and Voet 1995). Instead of being transported

through the blood-stream, eicosanoids are synthesised close to their site of action. (Voet and Voet 1995). The prime mode of eicosanoid action is through specific G protein-coupled receptors (GPCRs), several of which have been recently cloned (reviewed in (Narumiya, Sugimoto et al. 1999; Breyer and Breyer 2000; Funk 2001; Jacobs and Zeldin 2001). However, growing evidence suggests that these molecules play a dual role, not only acting in a paracrine way through GPCRs but also in an intracrine fashion via the activation of PPARs (reviewed in (Kliewer, Lehmann et al. 1999; Funk 2001; Nosjean and Boutin 2002). Consistent with this hypothesis, several different eicosanoids have been identified as ligands of PPARs (Section 1.4.1). In the case of 15d-PGJ2, several lines of evidence support its role as a ligand for PPARγ. 15d-PGJ2 is transported to the nucleus, it induces translocation of PPARγ to the nucleus, it binds nuclear proteins in whole cells and it enhances the binding of PPARγ to one of its coactivators (reviewed in (Nosjean and Boutin 2002).

1.5.3 Eicosanoids in *Drosophila*

Only a handful of studies have approached the function of eicosanoids in *Drosophila*. Early studies showed that arachidonic acid, the precursor of all eicosanoids discussed here is absent from *Drosophila* head extracts (Yoshioka, Inoue et al. 1985; Stark, Lin et al. 1993; Stark, Lin et al. 1993). In addition, the arachidonic acid-precursors, γ-linolenic acid (18:3) and 8,11,14-eicosatrienoic acid (20:3) are also undetectable (Pages, Rosello et al. 1986). However, pharmacological approaches have shown that phospholipase A2 inhibitors, which block eicosanoid biosynthesis, hinder the cellular immune response in *Drosophila*, suggesting a role for eicosanoids as cell-signalling molecules in the innate immune response (Carton, Frey et al. 2002; Yajima, Takada et al. 2003). Other pharmacological experiments blocking the synthesis of diacylglycerols, again affecting eicosanoid biosynthesis, suggest that polyunsaturated FAs may be intracellular messengers during photoreceptor excitation (Chyb, Raghu et al. 1999). Together, these studies suggest that although arachidonic acid is not the *Drosophila* precursor of active VLCFAs, several pathways leading to the formation of VLCFA-like molecules are conserved.

1.5.4 Eicosanoid Synthesis

All vertebrate eicosanoids are derived from a C20:4 VLCFA named arachidonic acid (5,8,11,14-eicosatetraenoic acid), which itself is synthesised from linoleic acid by elongations and desaturations. Arachidonic acid is not always synthesised *de novo*, but can be stored in cell membranes, esterified to phosphatidylinositol, other phospholipids and diacylglycerols (Figure 1.4, reviewed in (Voet and Voet 1995; Berg, Tymoczko et al. 2001). There are two main pathways of arachidonate metabolism: the cyclic pathway and the linear pathway. The cyclic pathway leads to the formation of the cyclopentane ring characteristic of prostaglandins. The first step of this pathway involves the formation of prostaglandin H2 (PGH2) by the action of prostaglandin G/H synthases (PTGS1 and PTGS2). Other prostaglandins and the highly-related molecules prostacyclins and thromboxanes arise from PGH2 by the action of downstream cytochrome P450 enzymes (P450s) coordinated in a cell-specific manner. The linear pathway leads to the formation of HETEs, HPETEs and leukotrienes also via reactions catalysed by P450s (reviewed in (Voet and Voet 1995; Berg, Tymoczko et al. 2001).

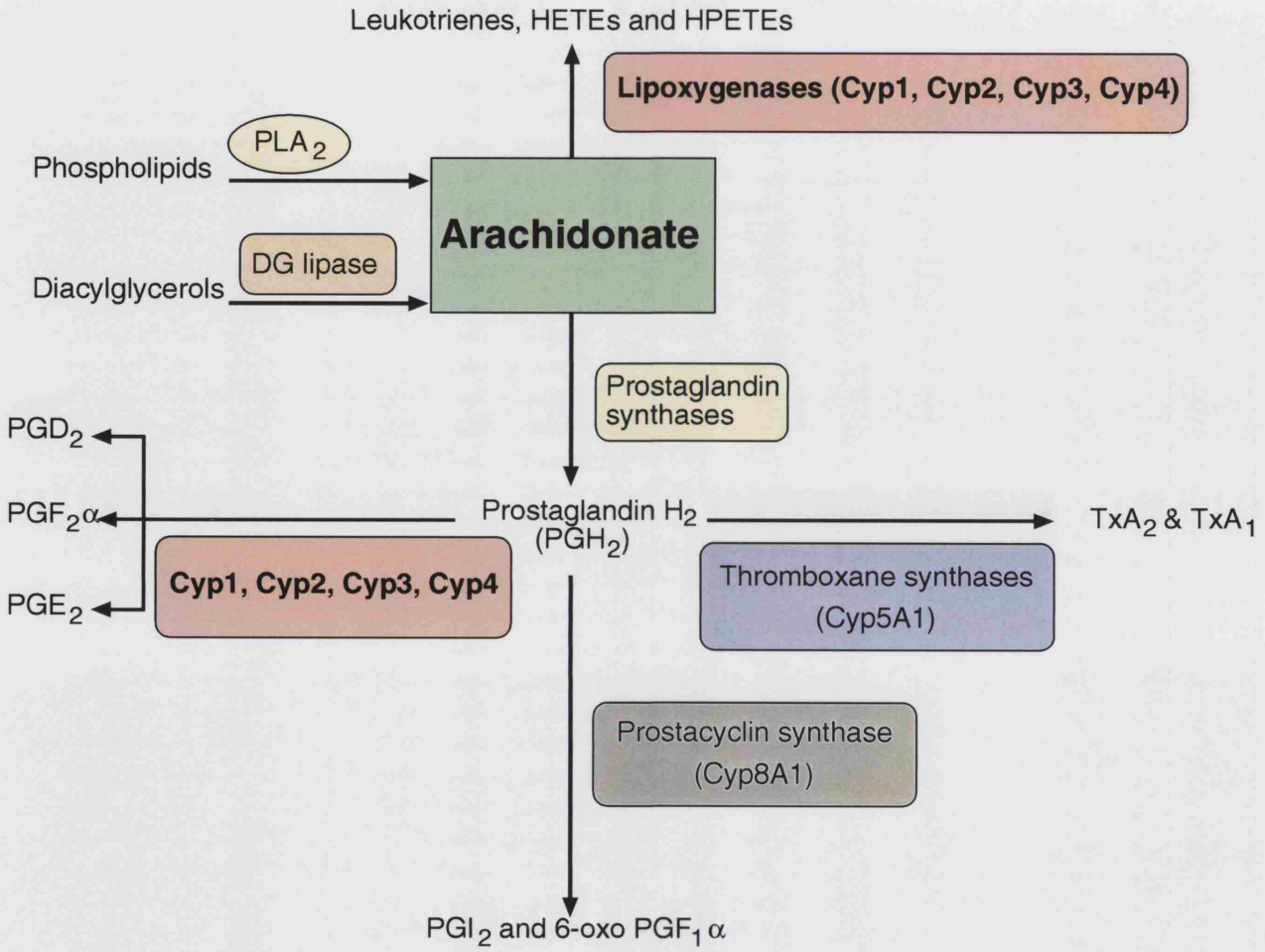
1.5.5 Role of Cyp4 Cytochrome P450s in Eicosanoid Synthesis

Eicosanoid production is region-specific, with different tissues producing different variants. The super-family of cytochrome P450 enzymes (Cyps) have been implicated in this tissue specificity. P450 enzymes are mainly known for their ability to metabolise xenobiotics. Nevertheless, all cytochrome P450 enzymes probably have at least one endogenous substrate. For example, eight families of vertebrate P450s are involved in the epoxidation, reduction and oxidation of arachidonic acid derivatives to yield more than 102 different eicosanoid metabolites (Figure 1.4, reviewed in (Nebert and Russell 2002).

The Cyp4 family of microsomal cytochrome P450s has been implicated almost exclusively in the metabolism of FAs, including those that are arachidonic acid-derived (Figure 1.4, reviewed in (Simpson 1997; Okita and Okita 2001). Cyp4 monooxygenases are capable of hydroxylating the terminal ω-carbon of certain eicosanoids and other FAs (Yamamoto, Kusunose et al. 1984; Yamamoto, Kusunose et al. 1984; Matsubara, Yamamoto et al. 1987; Sharma, Doig et al. 1989) and reviewed in (Simpson 1997; Okita and Okita 2001). The most frequent sites of vertebrate *Cyp4* expression are the liver and kidney, leading to the hypothesis that Cyp4 enzymes metabolise FAs in both of these tissues (Simpson 1997).

Figure 1.4 Eicosanoid Biosynthetic Pathway

Arachidonate is the common precursor for the synthesis of all vertebrate eicosanoids including linear (leukotrienes, HETEs and HPETEs) and cyclic eicosanoids (prostaglandins, prostacyclins and thromboxanes). Arachidonate is released from phosopholipids and diacylglycerols by Phospholipase A2 (PLA2) and Diacylglycerol lipase actions, respectively. Cytochrome P450 members of the Cyp4 family are involved both in the branch leading to linear eicosanoids (leukotrienes, HETEs and HPETEs) and in one of the branches leading to the formation of some of the cyclic prostaglandins (Modified from Voet and Voet 1995, Berg et al. 2001 and Nebert and Russel 2002).



Eicosanoid synthetic pathway
(modified from Nerbert & Russell 2002, Voet and Stryer 1998)

In vertebrates, there is a close association between PPAR activation, *Cyp4* induction, peroxisome proliferation and the induction of peroxisomal lipid oxidation. As mentioned above, PPARs are activated by dietary and endogenous ligands, some of which are believed to be produced by Cyp4 enzymes (Sharma, Lake et al. 1988; Sharma, Lake et al. 1988; Sharma, Doig et al. 1989; Simpson 1997). Once activated, PPARs trigger the transcription of downstream target genes involved in peroxisome proliferation and lipid degradation, including the *Cyp4* genes themselves (Sharma, Lake et al. 1988; Sharma, Lake et al. 1988; Issemann and Green 1990; Dreyer, Keller et al. 1993; Peters, Hennuyer et al. 1997; Cui, Kawashima et al. 2001), reviewed in (Simpson 1997; Chawla, Repa et al. 2001; Okita and Okita 2001). The activity of Cyp4 enzymes, coupled with cytosolic oxidation reactions, can produce long chain dicarboxylic acids (Kaikaus, Chan et al. 1993), which enter peroxisomes to be degraded via the ß-oxidation pathway.

In *Drosophila*, there are 83 apparently functional *P450* genes, of which 22 belong to the *Cyp4* family (reviewed in (Tijet, Helvig et al. 2001). Surprisingly, only a few P450 genes have been functionally characterised in detail in flies and none of these are of the Cyp4 class. Three genes (*dib, sad* and *shd*) are involved in the synthesis of ecdysone (Section 1.6.3, (Chavez, Marques et al. 2000; Warren, Petryk et al. 2002; Petryk, Warren et al. 2003). Null mutations in any of the *Drosophila Cyp4* genes have yet to be described but different alleles of some have been isolated from insecticide-resistant and insecticide-susceptible strains (Dunkov, Rodriguez-Arnaiz et al. 1996). *Cyp4D1* has not been analysed at the functional level but it is known to be expressed throughout early development, peaking at late larval stages and decreasing very dramatically during pupariation. This observation led to the proposal that Cyp4D1 is required during late larval-early puparial stages (Gandhi, Varak et al. 1992). In addition, *Cyp4g15* expression is predominant in the brain cortex of third larval instar stage though its function here is not yet clear (Maibeche-Coisne, Monti-Dedieu et al. 2000). Two other *Cyp4* members studied in arthropods other that *Drosophila*, include crayfish *Cyp4c15* and cockroach *Cyp4c7*. Both of these cytochromes have been implicated in the synthesis of ecdysteroids and the suppression of juvenile hormone synthesis (Section 1.6.3 and Section 1.6.5). Summarizing, Cyp4-modified VLCFA-derived molecules can act as ligands of PPARs leading to the activation of a myriad of genes involved in lipid degradation.

1.6 Hormonal Pathways Coordinating Growth and Developmental Progression

However, lipid metabolism has to be coordinated, not only with nutritional status but also with growth, differentiation and developmental progression. Networks of specialized tissues, interconnected by hormonal signals, have evolved to achieve this coordination between nutrition, growth and developmental progression. In insects and vertebrates, insulin signalling plays an important role in coordinating cell size and division, organ size and also overall body size (reviewed in (Oldham, Bohni et al. 2000; Stocker and Hafen 2000; Nijhout 2003; Stern 2003). In addition, two classes of lipid hormones have been linked with developmental progression: ecdysteroids and juvenile hormones (JHs, reviewed in (Riddiford 1996; Lafont 2000).

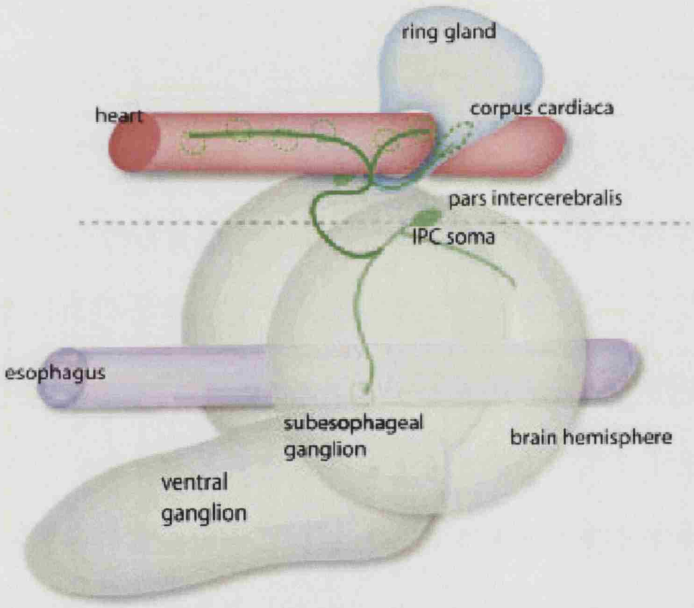
1.6.1 The *Drosophila* Insulin Pathway Regulates Growth and is Linked to Developmental Progression Via Secondary Hormones

Many animals can switch between a reproductive mode, when favourable conditions are present, and a slow-aging mode such as the dauer state in *C. elegans*, diapause in *Drosophila* or hibernation in some vertebrates (Carey, Liedo et al. 1998; Tatar, Bartke et al. 2003). During the reproductive mode, most energy is channelled towards reproductive processes, including increased metabolism, germ cell maturation and reproductive behaviours, all at the expense of stress-response mechanisms. During the slow-aging state, however, metabolism is decreased and stress responses are increased. Insulin-like peptides have been shown to play an important part in the regulation of such events. In invertebrates, food intake is integrated at the CNS level, where insulin-related signals are released and induce the production of secondary hormones that regulate metabolism, reproduction and aging by acting on germ-line and somatic tissues (reviewed in (Tatar, Bartke et al. 2003).

Drosophila insulin-like peptides (DILPs) are secreted by a group of seven median neurosecretory cells (m-NSCs), also known as insulin-producing cells (IPC), located in the brain lobes (Ikeya, Galic et al. 2002; Rulifson, Kim et al. 2002). These neurons extend projections into a part of the ring gland, the corpora cardiaca, and also into the heart and thus the hemolymph (Figure 1.5). The innervation of the corpora cardiaca has been suggested to induce JH production, which in turn stimulates egg follicle cells to produce ecdysone and makes somatic tissues, such as the fat body, modify their metabolism (Kozlova and Thummel 2000; Tu, Yin et al. 2002; Tatar,

Figure 1.5 IPCs Innervate the Corpora Cardiaca and Heart

Insulin-producing cells (green), located in the CNS, extend axons into the corpora cardiaca and heart of *Drosophila*. Dilps secreted into the corpora cardiaca may induce JH production, while Dilps distributed via the hemolymph may modify sugar metabolism (From Rulifson et al. 2000).


ring gland
heart
corpus cardiaca
pars intercerebralis
IPC soma
esophagus
subesophageal ganglion
brain hemisphere
ventral ganglion

Bartke et al. 2003). Nutritional availability has been shown to affect the expression of *dilp3* and *dilp5* genes in m-NSCs (Ikeya, Galic et al. 2002; Rulifson, Kim et al. 2002). In particular, circulating levels of tetrahalose and glucose are modified in m-NSC-ablated larvae, highlighting the role of these cells in sugar metabolism.

m-NSCs represent only one of several specialised cell-types that regulate energy metabolism in insect development. For example, a critical role is also played by the fat body, which produces endocrine signals that regulate neuroblast proliferation during larval development (Britton and Edgar 1998). More recently, the fat body has been proposed to act as an amino-acid sensor, hormonally regulating the growth of peripheral tissues by modulating insulin pathway activity, apparentrly in a m-NSC independent manner (Colombani, Raisin et al. 2003).

1.6.2 Insect Moulting as a Key Step in Developmental Progression

The insect body grows through a series of defined stages or instars that are separated by moults, that is, the shedding of the exoskeleton (cuticle, mouth structures and tracheal system). In hemimetabolous insects each instar increasingly resembles the adult both in terms of shape and size, the last moult rendering the organism competent to reproduce. In the more advanced holometabolous insects, the adult stage is preceded by a series of larval instars, where the body shape is radically different from the adult and is specialized for feeding. In such insects, the last moult is different from the rest and heralds the onset of metamorphosis, a process where the whole body plan is reshaped by histolysis of larval tissues, coupled with massive proliferation and differentiation of set-aside imaginal tissues that produces the fertile adult (Riddiford 1993). The different outcomes of larval-larval, larval-pupal and pupal-adult moults arise from the endocrine control that ecdysteroids and JHs exert on these processes.

One parameter that predicts the cessation of feeding and the commitment to undergo metamorphosis is what has been termed "critical size" (reviewed in (Nijhout 1994; Stern 2003). Experiments have suggested that individual organs grow until they attain a critical size and that attainment of this provides cues for other organs to stop growing. For example, tumorous over-proliferation of *Drosophila* imaginal discs produces larval arrests accompanied by excess growth (Sehnal and Bryant 1993). Conversely, lesions in imaginal discs delay entry into pupariation and this delay is proportional to the extent of the lesion and the extra growth (Simpson, Berreur et al. 1980). These experiments suggest that the time required to achieve a critical size, as

well as the value of this parameter can be modified, highlighting the connections between nutritional status, growth and developmental progression. Each insect moult is an elaborate process comprising a species-specific and stereotyped pattern of behaviour, excretion of digestive enzymes and secretion of hormones. Together, these events lead to the shedding of the old exoskeleton, clearance of tracheal airways and tanning of the new cuticle (Zitnan, Kingan et al. 1996; Kingan, Gray et al. 1997; Park, Filippov et al. 2002) reviewed in (Mesce and Fahrbach 2002). This complicated process is orchestrated by a series of peptide hormones ecdysis-triggering hormone (ETH), eclosion hormone (EH), crustacean cardioactive peptide (CCAP) and bursicon (reviewed in (Riddiford 1993; Mesce and Fahrbach 2002).

A variety of *Drosophia* genes give mutant phenotypes that include defects in moulting, such as duplicated mouth hooks, duplicated tracheal structures, duplicated cuticles and larval death. These have been classified into four groups, according to the level at which they act (Park, Filippov et al. 2002). Those affecting the synthesis and secretion of ecdysone include *ecdysoneless (ecd), defective in avoidance of repellents (dare)* and *IP-3 receptor (itpr*, (Belinski-Deutsch, Busson et al. 1983; Venkatesh and Hasan 1997; Freeman, Dobritsa et al. 1999). Genes acting in the ecdysone response, such as *ecdysone receptor B (EcR-B), ultraspiracle (usp), ßfushi tarazu factor 1 (ßftz-f1), ecdysone-induced protein E75A (E75A)* and *cryptocephal (crc*, (Perrimon, Engstrom et al. 1985; Schubiger, Wade et al. 1998; Hewes, Schaefer et al. 2000; Li and Bender 2000; Yamada, Murata et al. 2000; Bialecki, Shilton et al. 2002). Genes encoding peptide-processing enzymes, like *amontillado (amon)* or *peptidylglycine α-hydroxylating monooxygenase (phm*, (Gooding, Choksi et al. 2000; Jiang, Kolhekar et al. 2000), and finally genes acting within the ecdysis network, such as *ETH* (Park, Filippov et al. 2002).

1.6.3 Ecdysteroid Biosynthesis

In arthropods, ecydsteroids constitute one of the major hormones responsible for developmental progression including moulting (reviewed in (Riddiford 1993; Thummel 1995; Kozlova and Thummel 2000; Lafont 2000). In addition, ecdysteroids are required for oogenesis and subsequent embryonic development, when they are produced by extraembryonic tissue (amnioserosa) or by endocrine cells of the gonads, respectively (Kozlova and Thummel 2003); reviewed in (Hagedorn 1989; Lafont 2000). During *Drosophila* larval life, ecdysteroids are produced by a specialised set of

endocrine cells, known as the ring gland (Figure 1.5). This tissue releases two major ecydsteroids, α-ecdysone and 20-deoxymakisterone A, both of which are thought to be largely inactive (Riddiford 1993; Gilbert, Song et al. 1997). These ecdysteroids are formed from cholesterol but the exact biosynthetic pathway, starting from this precursor is not yet fully understood (reviewed in (Koolman 1990; Grieneisen 1994; Rees 1995). Recently, however, important advances have been made with three *Drosophila* P450 cytochromes identified as enzymes in the 20HE biosynthetic pathway by genetic and biochemical means (Figure 1.6, (Chavez, Marques et al. 2000; Warren, Petryk et al. 2002; Petryk, Warren et al. 2003). All three genes encoding these P450s are members of the Halloween family of embryonic lethal mutants that fail to produce cuticle. The first two, *disembodied (dib)* and *shadow (sad)* catalyse the last two steps of the ecdysone biosynthetic pathway. Accordingly, both are expressed in sites involved with ecdysone synthesis, such as the embryonic epidermis, larval ring gland and egg follicle cells. The conversion of ecdysone into its active metabolite, 20-hydroxyecdysone (20-E) occurs in many peripheral tissues, such as the epidermis, midgut, Malpighian tubules and fat body, by the action of the ecdysone-20-monooxygenase Cyp enzyme *shade (shd*, (Petryk, Warren et al. 2003). Very little is known about ecdysteroid catabolism, but the Cyp18 enzyme has been implicated as it is induced by ecdysone (Bassett, McCarthy et al. 1997). Cyp18 has also been suggested to encode a 26-hydroxylase, however, direct inactivation of 20HE has not yet been demonstrated.

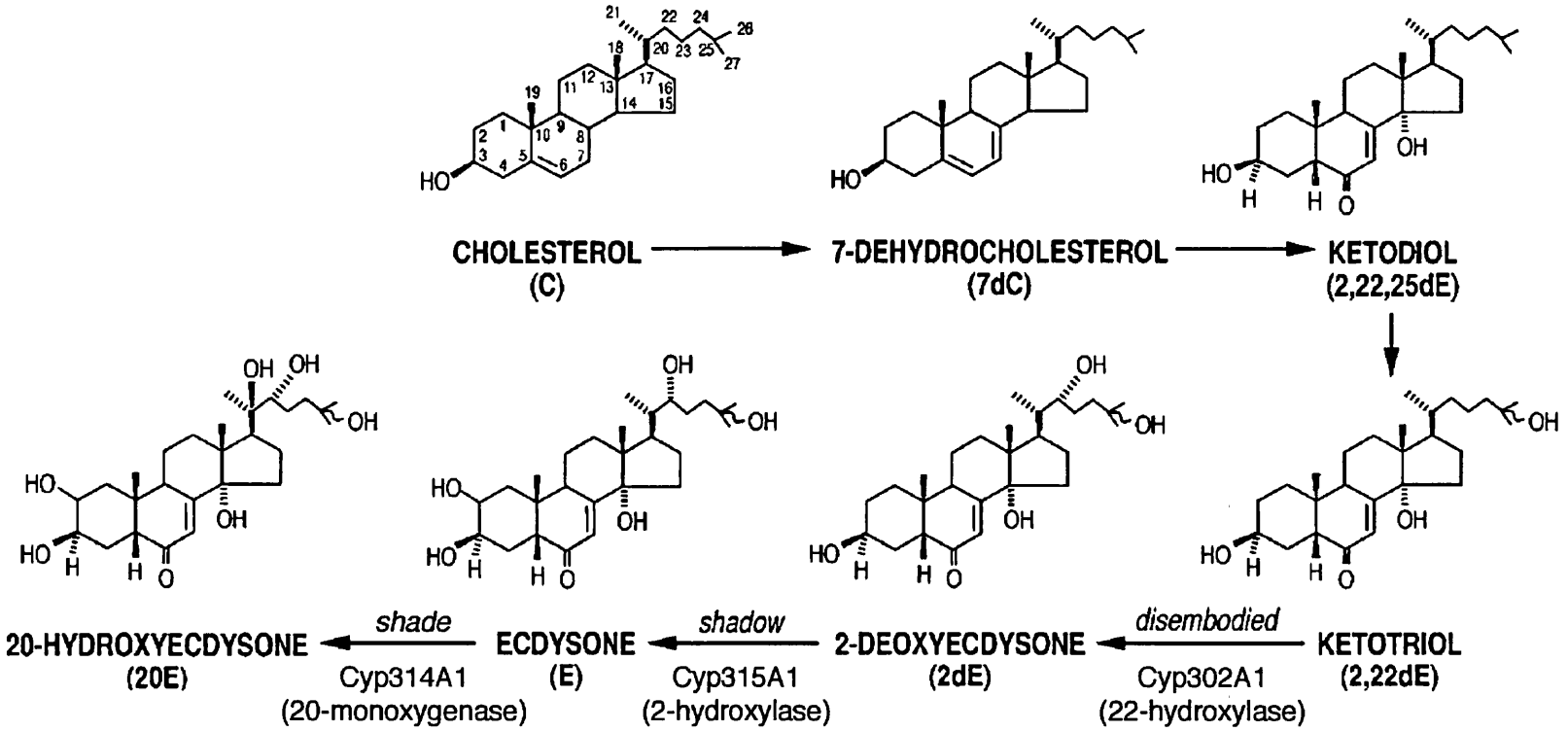
Ecdysteroid biosynthesis by the ring gland is highly regulated (reviewed in (Lafont 2000). Different neuropeptide inputs, are known to have long term effects on the ring gland, either stimulating or inhibiting ecdysteroid synthesis (Hua, Bylemans et al. 1994); reviewed in (Gade, Hoffmann et al. 1997; Lafont 2000). In addition, a combination of positive and negative feedback loops is believed to underly the appearance and disappearance of ecdysteroid peaks (reviewed in (Lafont 2000). However, the precise biosynthetic and catabolic enzymes mediating these feedback effects remain to be convincingly identified.

1.6.4 Ecdysteroid Signalling and its Role in Developmental Progression

Ecdysteroids function by specific binding to nuclear hormone receptors (reviewed in (Riddiford 1993; Thummel 1995; Kozlova and Thummel 2000; Lafont 2000). In fact, early studies on the effects of ecdysone on polytene chromosomes lead to the general

Figure 1.6 Three Cytochrome P450s Encoded by Halloween Group Genes are Involved in Ecdysone Biosynthesis

Proposed ecdysone biosynthetic pathway highlighting the steps for which three *Drosophila* enzymes have been characterized. Italics above arrows refer to the loci involved. Names below arrows refer to the cytochrome nomenclature name of the gene products and enzymatic activities are in brackets (modified from Warren et al. 2002).


CHOLESTEROL
(C)
7-DEHYDROCHOLESTEROL
(7dC)
KETODIOL
(2,22,25dE)
KETOTRIOL
(2,22dE)
disembodied
Cyp302A1
(22-hydroxylase)
2-DEOXYECDYSONE
(2dE)
shadow
Cyp315A1
(2-hydroxylase)
ECDYSONE
(E)
shade
Cyp314A1
(20-monoxygenase)
20-HYDROXYECDYSONE
(20E)

concept of ligand-receptor interaction, where steroid hormones act by regulating gene expression through their binding to intracellular receptors (Karlson 1963). The best characterised of these ligand-receptor couples is the one occurring between 20E and the Ecdysone Receptor (EcR), the *Drosophila* ortholog of vertebrate Liver X Receptor (LXR, (Lafont 2000; Maglich, Sluder et al. 2001). Several lines of evidence have shown that 20E binding to EcR leads to the activation of target genes (reviewed in (Riddiford 1993). There are three isoforms of EcR encoded by a single locus (Talbot, Swyryd et al. 1993). These share the same DNA-binding domain (DBD) and ligand-binding domain (LBD), but differ in their N-terminal transactivation domains. Tissue-specific expression patterns of the isoforms are, in part, responsible for the distinct ecdysone responses displayed by different tissues (reviewed in (Lafont 2000). EcR works by forming heterodimers with USP, another nuclear hormone receptor, encoded by *Ultraspiracle*, the *Drosophila* ortholog of vertebrate retinoid X receptor (RXR, (Thomas, Stunnenberg et al. 1993; Yao, Forman et al. 1993; Antoniewski, Mugat et al. 1996; Lezzi 1996; Hall and Thummel 1998; Wang, Miura et al. 1998). EcR-USP heterodimers bind to ecdysteroid response elements (EcREs) present in the promoters of target genes. These often resemble PPREs and contain either direct or inverted repeats of a 6 nucleotide half-site.

There is a higher degree of complexity in the ecdysteroid mode of action than might be suggested by the above 20E-EcR model. First, ligands other than 20E could potentially bind EcR. Second, USP can also be activated by ligand binding and another class of lipid hormones, JHs, have been implicated (Section 1.6.5). A third complexity is that, both EcR and USP can form heterodimers with other partners, such as EcR-Svp or DHR38-USP (reviewed in (Thummel 1995; Buszczak and Segraves 1998; Kozlova and Thummel 2000). In addition, many receptors and their partners are subject to phosphorylation, and this may modify their activities (Gilbert, Song et al. 1997; Rauch, Grebe et al. 1998). Finally, an EcR independent ecdysteroid signalling pathway, mediated by heterodimers of USP and DHR38, the ortholog of mammalian NGFI-B nuclear hormone receptors, has recently been described (Baker, Shewchuk et al. 2003).

The steps leading from ecdysteroid activation of nuclear hormone receptors to the coordinated processes involved in developmental progression are understood in some detail. Ecdysteroid peaks lead to the sequential activation of downstream genes, which can be classified as "early", "early-late" or "late" according to their time of

induction (reviewed in (Lafont 2000). There is a regulation between early and late genes as originally described in the Ashburner model of salivary gland puffing in response to 20HE (Ashburner, Chihara et al. 1974). Early genes encode a small set of transcription factors (E74, E75, BR-C, E93, E78B, DHR3, ß-FTZ-F1) that repress their own expression but at the same time induce the transcription of a large number of tissue-specific late genes (reviewed in (Riddiford 1993; Thummel 1995; Lafont 2000). These late genes are believed to play a more direct role in the behavioural and physiological changes leading to the reshaping of the insect body plan. Although some of these late genes, such as the glue genes, have been characterised, the identity of many remains unknown.

1.6.5 JH Biosynthesis, Mode of Action and Involvement in Developmental Progression

The juvenile hormones (JHs) comprise a group of sesquiterpene molecules produced by a subset of the endocrine cells of the ring gland, the corpora allata (Goodman 1990; Gade, Hoffmann et al. 1997). These hormones have been viewed as key players in the evolution of metamorphosis (reviewed in (Truman and Riddiford 2002). In basal insects, JHs can act to suppress embryonic growth while promoting nymphal differentiation; in holometabolous insects, these hormones are thought to maintain them in larval stages (reviewed in (Riddiford 1993; Riddiford 1996; Lafont 2000). In the most derived holomoetabolous groups, imaginal discs have escaped this JH morphogenetic suppression. Similar to ecdysteroids, JHs are also believed to have a gonadotropic role in adults (reviewed in (Jones 1995; Riddiford 1996; Hartfelder 2000; Tatar, Bartke et al. 2003). In most of insects, JH III (JHB3) represents the major JH component. In *Drosophila*, it has been postulated that a JH III bisepoxide is the major JH produced by the ring gland (Richard, Applebaum et al. 1989).

Part of the biosynthetic pathway leading to the formation of JHs has been elucidated. The initial steps on this pathway are common to those of the vertebrate cholesterol biosynthetic pathway and lead to the formation of farnesyl pyrophosphate. Then the pathway diverges towards JH-specific reactions (reviewed in (Lafont 2000). None of the enzymes involved in these last steps have been cloned in *Drosophila*. However, in cockroaches, one Cyp4 enzyme, Cyp4c7, having farnesol 12-hydroxylase activity and a sesquiterpenoid ω–hydroxylation activity. Cyp4c7 is believed to be involved in arresting JH production and it is possible that this occurs

via the activation of the insect FXR relative, EcR, in a similar way to how cholesterol synthesis is regulated in vertebrates by oxysterol-FXR interactions (Weinberger 1996; Sutherland, Unnithan et al. 1998; Kliewer, Lehmann et al. 1999; Lafont 2000).

Advances in understanding the mode of action of JHs have been difficult as these molecules show low-affinity binding to a great variety of proteins, including membrane-bound receptors, carriers, enzymes and nuclear receptors (reviewed in (Jones 1995; Riddiford 1996; Hartfelder 2000; Lafont 2000; Wheeler and Nijhout 2003). There is evidence that suggests that USP is a receptor for JHs (Xu, Fang et al. 2002); reviewed in (Lafont 2000; Tatar, Bartke et al. 2003). Another candidate JH receptor is the Methoprene-tolerant (Met) bHLH-PAS transcription factor (Ashok, Turner et al. 1998; Wilson and Ashok 1998; Pursley, Ashok et al. 2000).

1.7 The Oenocyte as a Subject of Investigation

Few studies have addressed the question of how lipid levels are regulated in insects. Fewer still have attempted to identify specialised lipid processing cell-types in insects. The larval oenocyte of *Drosophila* is a specialised cell-type that could be involved in these processes. The goal of my thesis studies is to identify the functions of oenocytes and I now outline what is already known about this rather mysterious cell-type.

1.7.1 Previous Studies on Larval Oenocytes

In *Drosophila*, two different generations of oenocytes have been described. Those formed during embryogenesis, which persist until late pupal life, are termed larval oenocytes. Those that are produced during metamorphosis, which persist throughout adult life, are called adult oenocytes. My project is concerned with the former type, the larval oenocytes, hereafter simply named oenocytes. These cells have recently been used as a model system for studying single cell-fate specification by *Hox* genes (Elstob, Brodu et al. 2001; Gould, Elstob et al. 2001; Brodu, Elstob et al. 2002). However, studies of these insect cells can be traced back much earlier to their initial discovery more than a hundred years ago (Landois 1865; Wielowiejski 1886). At this time, they were described as 'respiration cells', as individual air-filled tracheoles were observed to make connections with them (Landois 1865). Due to their morphology being typical of a secretory cell, they were also referred to as 'unicellular glands' (Graber 1873). It was Wielowiejski that first coined the term 'oenocytes' because of

the wine-yellow colour that these cells display in many insect species, although not in *Drosophila* where they are virtually colourless (Wielowiejski 1886).

In *Drosophila*, oenocytes are found in the first seven abdominal segments, but are absent from the head and thorax. They are clustered into groups of 4 to 8 cells that lie in a lateral position, just beneath the epidermis. They have been described in the winged orders of Hexapoda, the Pterygota but are apparently excluded from the non-winged insects, the Apterygota (Wheeler 1892; Snodgrass 1935). However, later studies in arachnids based on both anatomical and biochemical analyses, identified some cells at the base of legs as oenocytes (Romer and Gnatzy 1981). This later observation raises the, as yet unresolved, question of whether Apterygote insects might also possess oenocytes, perhaps in a location different from that in winged lineages. Both the number of segments bearing oenocytes and the number of cells per cluster are known to vary across species. A recent survey of the Pterygota literature revealed that oenocytes have been reported in seven or eight consecutive segments and the number per cluster varies anywhere from 4 to up to 50 cells (Wheeler 1892; Perez 1901; Glaser 1912; Eastham 1929; Thorpe 1930; Locke 1969; Rizki 1978). As many of these observations have relied on bright-field light microscopy, further studies with molecular markers are required to establish unequivocally which cells truly are oenocytes.

1.7.2 Oenocyte Formation and Early Differentiation

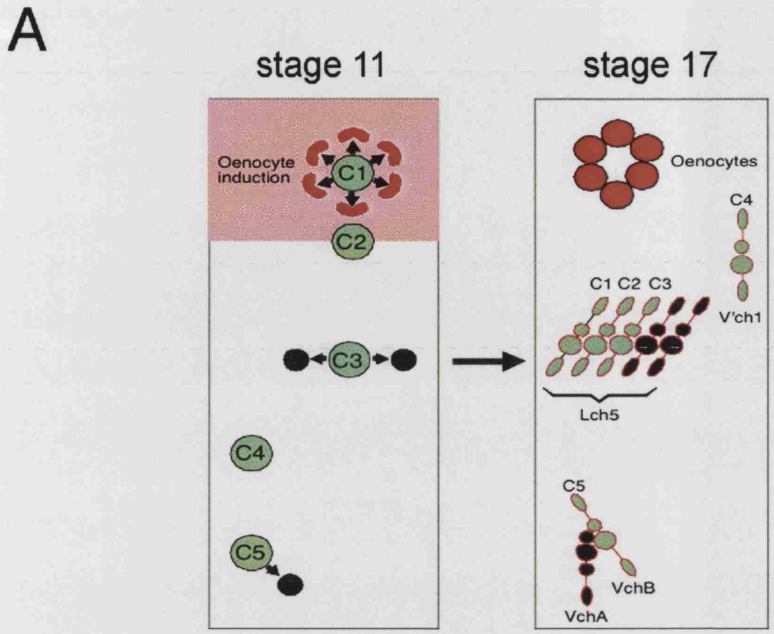
The embryonic origin of larval oenocytes has only been characterised very recently (Elstob, Brodu et al. 2001; Gould, Elstob et al. 2001; Rusten, Cantera et al. 2001; Brodu, Elstob et al. 2002). From these studies, it is clear that oenocytes provide a tractable single-cell model for addressing important biological questions, such as how *Hox* genes specify cell identity.

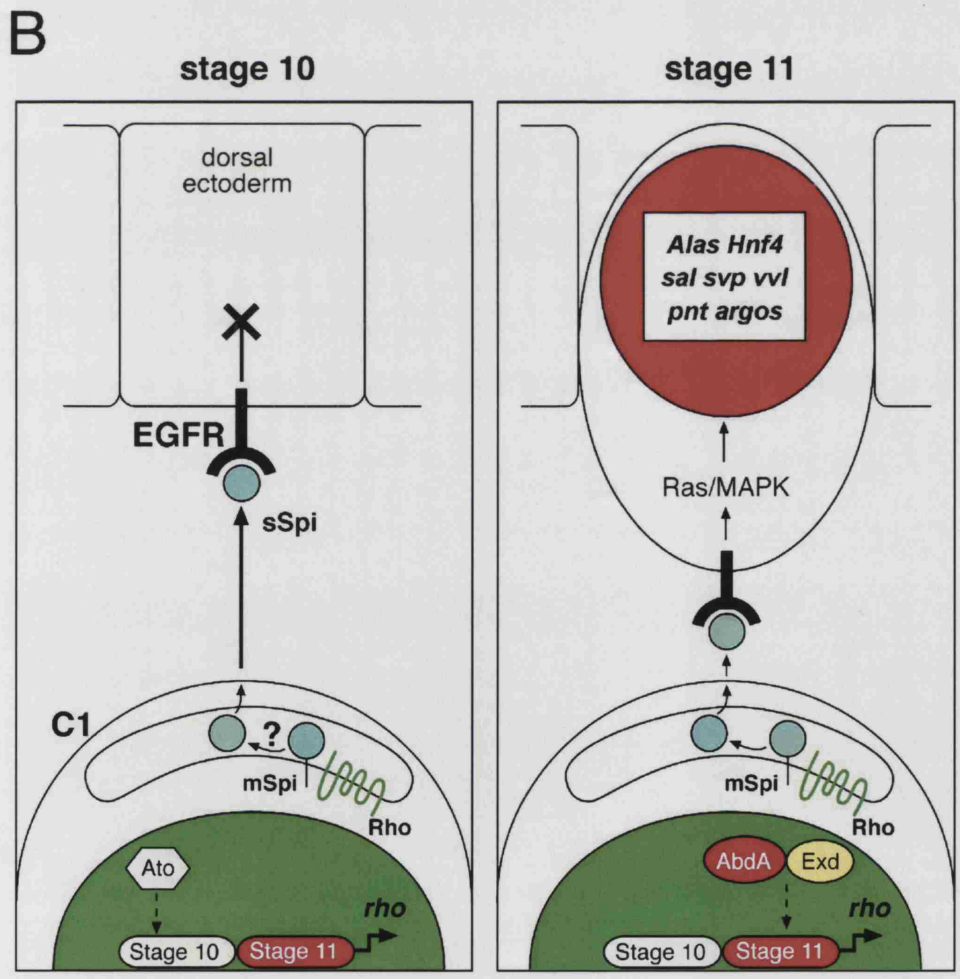
Oenocytes are induced from the dorsal ectoderm at the extended germ-band stage (Gould, Elstob et al. 2001). Recent genetic studies have provided insights into which molecules are involved in prepatterning the dorsal ectoderm to make it competent to form oenocytes in response to local induction (Elstob, Brodu et al. 2001; Gould, Elstob et al. 2001; Rusten, Cantera et al. 2001). More specifically, the paracrine signalling system involves two cell types: a chordotonal organ precursor cell (C1), which acts as the signalling cell, and the dorsal ectoderm which acts as the responding cell-type (Figure 1.7A). C1 signals to the dorsal ectoderm via the secreted

Figure 1.7 EGF Signalling from C1 to the Dorsal Ectoderm Induces Oenocytes in a Stage- and Segment-specific Manner

(A) Fate map for oenocytes and chordotonal organs. Each panel represent a single abdominal segment, oriented anterior to the left and dorsal up. The left panel illustrates 5 primary chordotonal organ precursors (C1-C5) at stage 11 and the induction of two alternative cell-types, oenocyte precursors or secondary chordotonal organ precursors, via EGF signalling (arrows). *spalt (sal)* expression (pink) provides a molecular switch that allows differentiation into the oenocyte fate as opposed to the secondary chordotonal organ fate. The right panel shows the derivatives of all precursor cells at stage 17. Lch5, V'ch1, VchA and VchB refers to the names given to anatomically distinct sets of chordotonal organs (From Gould et al. 2001).

(B) The EGF signal secreted Spitz (sSpi) is cleaved in the Golgi by the trnasmembrane protease Rhomboid (Rho) from its precursor, membrane-bound Spitz (mSpi). Maintenance of *rhomboid (rho)* expression through embryonic stage 11 is required for the signal to be present when the dorsal ectoderm is competent to respond to it. This maintenance is achieved only in abdominal segments expressing *abdominal A (abdA).* At stage 11, activation of EGFR signalling in the responding cell results in upregulation of *sal* and the induction of a series of downstream differentiation genes including: *seven-up (svp), ventral veins lacking (vvl), pointed (pnt), argos, Hepatocyte nuclear factor 4 (Hnf4)* and *delta-amino levulinate synthase (Alas,* Modified from Brodu et al. 2002).


A
stage 11
stage 17
Oenocyte induction
C1
C2
C3
C4
C5
Oenocytes
C4
C1 C2 C3
V'ch1
Lch5
C5
VchB
VchA


B
stage 10
stage 11
dorsal ectoderm
EGFR
sSpi
C1
?
mSpi
Rho
Ato
rho
Stage 10
Stage 11
Alas Hnf4
sal svp vvl
pnt argos
Ras/MAPK
mSpi
Rho
AbdA
Exd
rho
Stage 10
Stage 11

form of *Spitz (Spi)*, one of several *Epidermal growth factor Receptor (EGFR)* ligands in *Drosophila* (Freeman 1994; Tio, Ma et al. 1994; Okabe and Okano 1997; zur Lage, Jan et al. 1997). In turn, Spi ligand activates the *EGFR* pathway in the dorsal ectoderm, ultimately leading to the transcription of a series of oenocyte differentiation genes (Figure 1.7B). An early response to EGFR activation is the delamination and subsequent migration of the oenocyte precursor. Thus the final position of the oenocyte clusters, adjacent to the lateral chordotonal organs, is more ventral and anterior than the location at which they were induced.

One of the key molecules that prepatterns the responding ectoderm is a zinc finger transcription factor encoded by the gene *spalt (sal)*. Early *sal* expression is required in the dorsal ectoderm for it to become competent to form oenocytes upon *EGFR* activation (Figure 1.7A). More ventral regions of the ectoderm that do not express *sal* respond differently to the same *EGFR* activation, adopting an alternative cell fate, the chordotonal organ precursor. However, *sal* is not the only molecule responsible for prepatterning the dorsal ectoderm, as oenocytes can be formed only within a narrow stripe of cells expressing *engrailed*, a homeobox gene (Elstob, Brodu, Gould, unpublished). In addition, the *Notch* pathway blocks oenocyte induction during stage 10 (Brodu and Gould, in preparation).

1.7.3 Hox Genes and their Role in Oenocyte Formation

Many animals have a segmented body plan and the genes used to direct differences between segments are highly conserved throughout evolution. These consist of clusters of genes encoding homeodomain transcription factors that are called *Hox*/homeotic genes (McGinnis and Krumlauf 1992). Each of these genes has a specific expression domain along the anterior-posterior axis and directs morphological specialisations that make a segment different from its neighbours.

Traditionally, the role of *Hox* genes had been investigated at the level of a whole segment or organ. Studies on the formation of the haltere by the *Hox* gene *Ultrabithorax* concluded that *Hox* genes act as micromanagers, regulating the expression of a large number of different targets in a complex spatiotemporal manner (Akam 1998; Weatherbee, Halder et al. 1998). However, using the oenocyte system, it was possible to examine a *Hox* function at the single cell level (Figure 1.7B, (Brodu, Elstob et al. 2002); reviewed in (Lohmann and McGinnis 2002). Hox rescue experiments were used to show that *abdominal A (abdA)* triggers oenocyte induction

via the regulation of a single target gene, *rhomboid (rho)*, encoding a Golgi protease required to process *Spi* into its active form. The expression of *rho* within the C1 signalling cell is regulated in two distinct phases. A transient early phase of expression is activated at stage 10 (staging according to (Campos-Ortega and Hartenstein 1997) by the presence of Atonal, a BHLH transcription factor encoded by the proneural gene, *atonal*. Subsequently, maintenance of *rho* expression in the C1-lineage, specifically in the abdomen during stage 11 is mediated by *abdA*. This *rho* maintenance is required for all oenocyte induction as the dorsal ectoderm only becomes competent to respond appropriately at stage 11. The maintenance of Rho expression appears to be the only downstream function of *abdA* required for oenocyte induction and differentiation. These results raise the possibility that, although the overall number of *Hox* target genes may be large, on a per cell basis there may be very few direct targets. Hence, the multiple effects of *Hox* genes seen at the level of a segment may be the result of a complex multicellular mosaic of relatively simple changes in gene expression in individual cells. Clearly, this new view of Hox function requires testing in many more cell-types. It differs from the widely accepted viewpoint that the number of direct targets per nucleus is extremely large (Liang and Biggin 1998).

1.7.4 Main Hypotheses Concerning Oenocyte Function

In contrast to our expanding knowledge of oenocyte induction, the function of oenocytes has remained unresolved for over a century. As expressed by Glaser, 'no two people agreeing in more than a few points' (Glaser 1912). I will now outline three hypotheses for oenocyte function that have been proposed over the last one hundred and forty years.

1.7.4.1 Regulation of Hemolymph Composition

Some studies suggest that oenocytes regulate the composition of hemolymph. Koschevnikov postulated that unnecessary substances which get into the hemolymph stop in the interior of the oenocytes in the form of granules (Koschevnikov 1900), reviewed in (Glaser 1912). Janet favoured the idea that oenocytes take up substances from the circulating hemolymph, in turn metabolising them and transferring them to the fat cells for 'elaboration or dissolution' of reserves (Janet 1909). Electron microscopical analysis of the structure of the ER suggested that oenocytes might be

involved either in detoxification or steroid metabolism (also see Section 1.7.4.2; (Locke 1969). More recently, *Drosophila delta-aminolevulinate synthase gene (Alas)* was cloned and found to be expressed within oenocytes (Ruiz de Mena, Fernandez-Moreno et al. 1999). This gene encodes an enzyme required for the biosynthesis of heme, a prosthetic group attached to cytochrome P450s and other hemoproteins. As cytochrome P450s are known to act in xenobiotic detoxification processes, it has been proposed that oenocytes may facilitate the removal of toxins from the hemolymph (Ruiz de Mena, Fernandez-Moreno et al. 1999).

1.7.4.2 Involvement in Oxygen Sensing

Anatomical connections with the tracheal system suggested that oenocytes might participate in the respiration process (Landois 1865; Wheeler 1892). In addition, pioneering physiological experiments involving a colorimetric reaction, using phenolphthalein and hydrogen peroxide added to oenocyte extracts of leopard moth (*Zeuzera pyrina*) larvae, revealed the presence of oxidising enzymes, such as oxidases or catalases (Glaser 1912). Interestingly, more recently it was found that the *Drosophila* homologues of two vertebrate genes involved in the hypoxic response (Guillemin and Krasnow 1997) are expressed in oenocytes. These genes are *Hnf-4* and *seven-up (svp*; (Hoshizaki, Blackburn et al. 1994; Ruiz de Mena, Fernandez-Moreno et al. 1999). The vertebrate ortholog of *dHnf-4* has been shown to activate the transcription of the erythropoietin gene *(EPO)* in response to hypoxia in the liver and kidney. The vertebrate homolog of *svp*, *EAR3/COUP-TF1*, plays an opposite role, repressing *EPO* transcription (Galson, Tsuchiya et al. 1995). In addition, as mentioned above, *Alas* is required for the synthesis of heme and some vertebrate hemoproteins, such as hemoglobin, are known to act as oxygen carriers. Moreover, heme itself serves as an oxygen sensor (Labbe-Bois and Labbe 1990).

1.7.4.3 Involvement in the Moulting Process

Morphological studies and *in vitro* experiments have suggested that oenocytes may be involved in the moulting process in one of two possible ways: either by producing components of the cuticle in response to 20-E (Wigglesworth 1933; Wolfe 1954; Wigglesworth 1970) or by synthesising the moulting hormones themselves (Koller 1928; Locke 1969; Romer, Emmerich et al. 1974; Studinger and Willig 1975; Dorn and Romer 1976; Romer and Gnatzy 1981). Early studies by Hollande found that

oenocytes store deposits of wax in their cytoplasm and were therefore renamed as 'cerodycytes' (Hollande 1914). Hollande also observed that this wax disappeared after fasting or during metamorphosis. Experiments in which abdomens of houseflies were incubated with labelled cholesterol have shown that these preparations are able to produce ecdysone and 20-E (Studinger and Willig 1975). Additionally, in these experiments, oenocytes become intensively labelled, suggesting a role for this cell-type in ecdysteroid synthesis. Similarly, when prothoracic glands of the mealworm beetle, *Tenebrio* molitor, are incubated with radiolabelled cholesterol, they produce labelled ecdysone, but if prothoracic glands are co-cultured with oenocytes, 20-E is produced, suggesting that oenocytes may be a site for ecdysone to 20-E conversion (Romer, Emmerich et al. 1974). Nevertheless, if an excess of unlabelled ecdysone is not are added to the co-cultures, labelled cholesterol is transformed into polar conjugates. This argues that ecdysone to 20-E conversion by oenocytes only occurs when the system is saturated and not at more physiological levels.

One day after pupariation, the prothoracic gland degenerates (Dai and Gilbert 1991) and it is not known which tissues functionally replace it to produce the major pupal peak of ecdysteroids that is known to trigger adult metamorphosis. These observations are intriguing as the oenocytes are the last larval tissue to undergo apoptosis during pupal life, raising the possibility that they may provide the major pupal source of ecdysteroids (Gould, unpublished).

In summary, there is some evidence consistent with each of the three hypotheses put forward for oenocyte function. These hypotheses are not mutually exclusive and, as they are based largely on circumstantial evidence, more experiments are required to test each one rigorously.

1.8 Conclusions

The understanding of lipid metabolism and how it is regulated according to nutritional status is much more advanced in vertebrates than in *Drosophila*. In vertebrates, PPARs are known to play a central role in regulating lipid homeostasis and recently Cyp4 hydroxylation of VLCFAs has been implicated in the formation of endogenous PPAR ligands.

The use of a genetically tractable and fast-developing system such as *Drosophila* could provide several for studying lipid metabolism. Previous work has

suggested that insect oenocytes might be involved in lipid metabolism and/or lipid signalling. The subject of my thesis is to investigate this issue in detail, with the aim of determining the *in vivo* function of oenocytes. To achieve this goal, I have taken two complementary approaches. One focuses at the cellular level, asking what is the phenotype resulting from the ablation of oenocytes. The second one is more molecular, identifying gene products expressed in oenocytes, with the hope that they will provide insights into the types of biochemical processes occurring within oenocytes.

CHAPTER TWO

Materials and Methods

CHAPTER TWO: Materials and Methods

2.1 *Drosophila* Stocks

All crosses were done at 25 °C on standard cornmeal/agar food.

The following loss-of-function alleles were used: hnt^{E8} (Strecker, Yip et al. 1991), svp^{e22} (Gausz, Gyurkovics et al. 1981), sal^{16} and sal^{A405} (Kuhnlein, Frommer et al. 1994). *svp-lacZ* refers to svp^{don1} enhancer trap (Elstob and Gould, Flybase, 1999). Two oenocyte-specific reporter lines were used: *BO-lacZ* (Barrio, de Celis et al. 1999) and *svp2.9-lacZ* (R Schultz, University of Texas, MSA); the latter contains a 2.9kb fragment of the promoter region of *svp* fused to *lacZ*. OK72 (C. O'Kane, University of Cambridge, UK) and 56B (A. Brand, Wellcome/CRC Institute, Cambridge, UK) refer to *GAL4* enhancer trap lines. UR5S refers to a *UAS-rpr* line with five copies of this transgene recombined onto chromosome III (A. Brand, Wellcome/CRC Institute, Cambridge, UK).

The following enhancer trap *lacZ* lines were obtained from the Bloomington Stock Center: 10538, 10706, 10880 ($mirr^{cre2}$), 10890, 10898, 11340 (sal^{03602}), 11342 ($Aldh\text{-}III^{03610}$), 11785, 11963, 12103, 12139, 12247, 12356, together with two *UAS-rpr* lines (stocks 5823 and 5824), two *UAS-lacZ* lines (stocks 3956 and 5148) and one *UAS-mCD8:GFP* (stock 5130). *GFP* enhancer trap line L14A2 was a gift from Bruno Bello and Lydia Michaut. The *GAL4* enhancer trap lines P0197 and P0206, as well as the *lacZ* enhancer trap lines P0030, P0093, P0103 and P0110 were obtained from Umeå Drosophila Stock Center.

For a complete genotype of these lines, refer to Table 2.1.

2.2 Recovery of Sequences Flanking P-element insertions by Inverse PCR

All molecular biology was done according to standard techniques (Sambrook, Fritch et al. 1989), unless otherwise stated. Sequences flanking the transposable element of each line were recovered as previously described:

(Rehm EJ, 2001, BDGP, http://www.fruitfly.org/p_disrupt/inverse_pcr.html).

Briefly, DNA was extracted from adult flies, digested either with Sau3A I or Msp I and religated. Flanking sequences were amplified by inverse PCR with the following primers: PGAL4 (5'-CTG CAG AAG CTT CAA GCC TCC TGA AAG-3', forward primer) and kp53 (5'-ATA CTT CGG TAA GCT TCG GCT ATC GAC G-3', reverse primer) were used for 5' ends of lines carrying pGawB (54°C annealing temperature); Plac4 (5'-ACT GTG CGT TAG GTC CTG TTC ATT GTT-3') and

Table 2.1 – Fly Stock Genotypes

10538 (y^1 w^{67c23}; P{w^{+mC}=lacW}l(2)k04003^{k04003}/CyO)

10706 (y^1 w*; P{w^{+mC}=lacW}C3-2-2)

10880 (y^1 w*; P{w^{+mC}=lacW}mirr^{cre2}/TM3, Sb^1)

10890 (y^1 w*; P{w^{+mC}=lacW}l(3)A6-3-56^1/TM3, Sb^1)

10898 (y^1 w*; P{w^{+mC}=lacW}B4-2-27/CyO)

11340 (P{$ry^{+t7.2}$=PZ}salm^{03602} cn^1/CyO; ry^{506})

11342 (P{$ry^{+t7.2}$=PZ}Aldh-III^{03610} cn^1/CyO; ry^{506})

11377 (cn^1 P{$ry^{+t7.2}$=PZ}dap^{04454}/CyO; ry^{506})

11785 (P{$ry^{+t7.2}$=PZ}ms(3)72D^{03957} ry^{506}/MKRS)

11963 (w^{67c23} P{w^{+mC}=lacW}l(1)G0273^{G0273}/FM7c)

12103 (P{$ry^{+t7.2}$=PZ}l(3)rL061^{rL061} ry^{506}/TM3, Sb1)

12139 (w^{67c23} P{w^{+mC}=lacW}l(1)G0122^{G0122}/FM7c)

12247 (w^{67c23} P{w^{+mC}=lacW}l(1)G0359^{G0359}/FM7c)

12356 (P{$ry^{+t7.2}$=PZ}l(2)08717^{08717} cn^1/CyO; ry^{506})

B032-*lacZ* (Barrio et al., 1999)

hnt^{E8} (y^1 peb$^{hnt\text{-}E8}$/FM7c, stock 3241) (Strecker et al., 1991)

l(2)05730 (Samakovlis et al., 1996)

L14A2 (Lydia Michau, unpublished)

OK72 (C. O'Kane, University of Cambridge, UK)

P0030 (P{lArB} ; ry^{506}; SW084-A7.10 X W, Claudia Sachs, diploma work 1990)

P0093 (w^1;P{$RY^{+t7.2}$=lArB};ry^{506}(1;3);MW057-130a)

P0103 (yw P{lacW}; ES012 - 65.12.1)56B (Brand and Perrimon, unpublished)

P0110 (y^1w^1 P{w^{+mC}=lacW};ES074-69.35.2)

P0197 (w^1;P{w^{+mC}=GAL4}162.1/CyO)

P0206 (w^1;P{w^{+mC}=GAL4}1018.1(2LR))

sal^{16} (Kuhnlein et al., 1994)

sal^{A405} (Kuhnlein et al., 1994)

svp-2.9kb (R. Schultz, The University of Texas M.D. Anderson Cancer Center, Houston, Texas)

svp^{don1} (Elstob and Gould, 2000)

svp^{e22} (Gausz et al., 1981)

UAS-mCD8:GFP (y^1 w*; Pin^{Yt}/CyO; P{w^{+mC}=UAS-mCD8::GFP.L}LL6, stock 5130)

UAS-nls-lacZ (*w^{1118};; P{w^{+mC}=UAS-lacZ.NZ}J312*, stock 3956)

UAS-rpr (w^{1118}; P{w^{+mC}=UAS-rpr.C}14, stock 5824)

UAS-rpr (w^{1118} P{w^{+mC}=UAS-rpr.C}27, stock 5823)

UAS-tau-lacZ (*y^1 w*; P{$y^{+mDint2}$=UAS-lacZ.βtau.YES}LL8/CyO*, stock 5148)

UAS-rpr^{UR5S} (w;; 5x P{w^{+mC}=UAS-rpr}/TM3, Sb, e) (A. Brand, Wellcome/CRC Inst., Cambridge, UK)

kp53 were used for 5' ends of lines carrying *pPZ, pLacW* and *plArB*; Pry1 (5'-CCT TAG CAT GTC CGT GGG GTT TGA AT-3') and Pry2 (5'-CTT GCC GAC GGG ACC ACC TTA TGT TAT T-3') were used for all 3' ends. PCR products were cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen) and sequenced using standard methods.

2.3 *In silico* Identification of Oenocyte-Genes

The Berkeley Drosophila Genome Project (BDGP) *in situ* database was used to search for genes expressed in oenocytes during embryonic stages. This search was performed using a web-based interface (http://www.fruitfly.org/cgi-bin/ex/basic.pl). Predicted products for these genes were subsequently compared on protein databases by use of the BLASTP tool (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4 Generation of *Oenocyte Enhancer-Gal4* Transformants

All the enhancer elements, except *BO-pC4G4,* were cloned into the recipient *GAL4* transforming vector *pWHS* (C. Cenci and A. Gould, unpublished). Briefly, this was built by fusing *hsp70* basal promoter coming from *pSKTH* and the *GAL4* coding sequence coming from *pGaTB* into the backbone of *pWHZ.* The resultant vector, *pWHS,* has a multicloning site upstream of the *hsp70* fragment containing unique sites for EcoR I, Bgl II, Not I, Ksp I and Kpn I. To construct *BO-pWHS,* a 7.6kb region driving oenocyte-specific expression, was released from *BO-pC4PLZ* (Barrio, de Celis et al. 1999) by EcoR I digestion and cloned in the same orientation into the EcoR I site of *pWHS.* To construct the *svp-pWHS* series, a *svp* 3kb enhancer element expressed in oenocytes and ring gland, localised 4.4kb upstream of the *svp* promoter (RA Schulz, unpublished), was released from *pP{CaSpeR-lacZ}* by digestion with EcoR I. This 3kb element was cut with Sma I to create a 1.7kb and a 1.3kb fragment. In a subcloning step, both pieces were cloned into *pBluescript II KS* (Stratagene) previously digested with EcoR I and Sma I to form *svp1.7-pBS* and *svp1.3-pBS.* The *svp* 1.7kb fragment was then released by EcoR I and Spe I digestion and cloned into *pWHS* digested with the same two enzymes. The *svp* 1.3kb fragment was excised from *svp1.3-pBS* by Kpn I and Not I digestions and re-cloned into *pWHS* cut with the same two enzymes. To make *svp0.4-pWHS, svp1.3-pBS* was digested with BsrG I and EcoR I, blunted with Klenow, and the larger fragment purified. This was then

self-ligated, producing the smaller *svp0.4-pBS* plasmid. The *svp* 0.4kb element was released by Not I and Kpn I digestion and ligated into *pWHS* cut with the same two enzymes to produce *svp0.4-pWHS*. *svp0.9-pWHS* was made in a similar way to *svp0.4-pWHS* but digesting with Sma I instead of EcoR I.

The *pC4G4* cloning vector, containing a P-element transposase basal promoter fused to the *GAL4* coding region, was constructed to try to mimic, as closely as possible, the structure of the promoter region of the highly oenocyte-specific *BO-pC4PLZ* construct (Barrio, de Celis et al. 1999). The P-element *pGawB* vector (Brand and Perrimon 1993; Brand, Manoukian et al. 1994) was digested with EcoR I, Klenow filled-in and self-ligated to destroy this restriction site. Then, to release a 3.5kb fragment containing both the P transposase basal promoter and the *GAL4* coding sequence, *pGawB* was digested with BamH I, subjected to a 2-base pair fill-in reaction and a BstZ17 I digestion. *pCaSpeR-4* (Thummel and Pirrotta 1991) was digested with Xho I, 2-base pair filled-in and Hpa I digested. The 3.5kb promoter-*GAL4* element was cloned into this *pCaSpeR-4* recipient, giving the *pC4G4* vector. This vector contains unique sites for EcoR I, Kpn I, Sac II, Sfi I and BamH I. To make *BO-pC4G4*, the *BO* element released from *BO-pC4PLZ* by EcoR I was cloned into the same site in *pC4G4*.

2.5 Generation of *UAS-RNAi* Transformants

To generate inducible intron-spliced snapback RNA interference lines, PCR fragments of *Cyp4g1, CG18609, CG11151, Hnf4* and *svp* were cloned as inverted repeats into the *UAS*-RNAi vector pWIZ (Lee and Carthew 2003). cDNA clones were used as templates (GH05567 for *Cyp4g1,* RE06553 for *CG18609,* RE42326 for *CG11151,* RE09535 for *Hnf4* and RE08410 for *svp*) and amplified with Pfu polymerase, except for the case of *Hnf4*, where Taq polymerase was used. The following pairs of 5' tagged primers were used: 5'-GAG TAC TAG TAA GAG GAG TCA CGT GCG ATT GTT G-3' and 5'-GTT GAC TAG TGC GAA GAC TTT AGC CTG GAT G-3' (330 bp *Cyp4g1* fragment); 5'-TCT ATC TAG AGT GGC CAG CGG TTC CTC A-3' and 5'-CTG GGC TAG CGT AGA CCT TCA AGA CCG TTT TCA A-3' (224 bp *CG18609* fragment); 5'-TCA TTC TAG ACG ACC ATC CCG CTC CCA CAC-3' and 5'-CAA CCG CTA GCC CTC GTT CTC CTT CAG TCC ATC-3' (214 bp *CG11151* fragment); 5'-AGT GTC TAG AGG CGG GCA TGA AGA AGG AGG CG-3' and 5'-AAT ATC TAG ACA GAA CCG GCA GGA TGA

GCA GAA TC-3' (720 bp *Hnf4* fragment); 5'-ACA CAC TAG TCG CTG CGA ACG GTC TCC TCA CAA G-3' and 5'-TGC TAC TAG TGC TGC CGC CGC TGC CGT ATG-3' (406 bp *svp* fragment).

All the constructs were introduced in *yw* hosts by P-element-mediated transformation using standard methods (Roberts 1998).

2.6 Histochemical Detection of ß-Galactosidase and Immunostaining

Embryo immunostaining and ß-galactosidase detection in larval tissues were according to standard protocols using HRP (Vector labs), Alexa fluorescent conjugates (Mol. Probes) or X-gal (Invitrogen). Primary antibodies used were: Mouse 22C10/anti-FUTSCH (gift of Seymour Benzer) at 1:20, Mouse 1G9/anti-HNT (DSHB) at 1:20, Mouse 2A12/anti-tracheal (DSHB) at 1:10, Mouse EcR-A (Talbot, Swyryd et al. 1993) at 1:10, Rabbit anti-ß-gal (Cappell) at 1:6000, Rat 9F8A9-ELAV antibody (DSHB) at 1:50, Mouse 433-43-2e/anti-Cyp4g1 (gift of Steve Kennel) at 1:1000 and Mouse anti-Hnf4 (gift of Marita Buescher) at 1:5000.

2.7 *In Situ* Hybridisation

Whole-mount *in situ* hybridisations were performed as described (Alexandre, Lecourtois et al. 1999). The *alas* probe was synthesised as described previously (Ruiz de Mena, Fernandez-Moreno et al. 1999). *Hnf-4* anti-sense RNA was prepared by linearizing the SD08077 cDNA (BDGP) with EcoR V and transcribing with SP6 RNA polymerase to produce a 1kb RNA. *CG11151* anti-sense RNA was made by linearizing the LP03652 cDNA (BDGP) with EcoR V and transcribing with SP6 RNA polymerase to produce a 0.8kb RNA.

2.8 Lethality and Hormone Rescue Experiments

Ecdysteroid feeding experiments were performed as described (Bialecki, Shilton et al. 2002), modifying the exposure time from a 6 hour pulse at late L2 to a continuous larval regime. Newly hatched larvae were collected for a period of 3 hours and immediately transferred to agar plates containing yeast with either 3.3% ethanol (50mg dry yeast, 95ml water and 5ml 100% ethanol), 0.33mg/ml 20-hydroxyecdysone in 3.3% ethanol (50mg dry yeast, 95ml water and 5ml of 10 mg/ml 20-hydroxyecdysone [Sigma] in 100% ethanol) or 0.66 mg/ml ecdysone [Sigma] in 3.3% ethanol. The yeast paste was replaced daily and in the case of those genotypes

and treatments where larvae developed to wandering L3, they were transferred when they reached this stage to vials of standard cornmeal/agar food covered with a layer of yeast paste containing the appropriate concentration of hormone. The following genotypes were assayed for lethality: *yw* x *UAS-rpr[5823]* (negative control), *snaP0206* x *UAS-rpr[5823]* (positive control for ablation of ring gland) and *BO-GAL4EG79.7* x *UAS-rpr[5823]* (oenocyte-less experimental animals).

CHAPTER THREE

Oenocyte Ablation as an Approach to Understand Oenocyte Function

"The function of the cells known as the oenocytes has been the object of a considerable amount of speculation by various investigators."

Glaser, R.W., 1912.

CHAPTER THREE: Oenocyte Ablation As An Approach To Understand Oenocyte Function

3.1 Establishment of a Genetic System to Ablate All Oenocytes

Ablation is a classical approach, often used in developmental biology to attribute functions to particular organs, tissues or particular cell-types. In the past, ablations were carried out by mechanical surgery, laser ablation or the use of selective chemical toxicity. To attempt to elucidate the function of oenocytes in third instar larvae (L3), laser ablation was used (A. Gould, unpublished). Its efficiency was assessed by means of a GFP reporter line expressed in oenocytes. However, this method proved to be very time consuming and, more importantly, did not permanently remove the very large larval oenocytes (50μm diameter), as seen by the recovery of the reporter expression after a few hours. The toxin Ricin was also used, crossing a *UAS-ricin* line with a driver (P0206) expressed in oenocytes. However, even though Ricin A-chain is very toxic at low concentrations (Sentry, Yang et al. 1993; Hidalgo, Urban et al. 1995; Greenspan 1997), its efficiency could not be so easily measured, as the expression of Spalt or GFP protein was not modified in embryonic oenocytes expressing *UAS-ricin* (A. Gould, unpublished). Relatively recent advances in our understanding of the *Drosophila* apoptotic pathway, have led to the addition of one more tool to the ablation kit: the *UAS-rpr* system, by which one can supply a pro-apoptotic protein with spatio-temporal control to artificially induce cell death (Aplin and Kaufman 1997). This system has now been used successfully in several instances (see for example (Ikeya, Galic et al. 2002; Lohmann, McGinnis et al. 2002). Unlike with the Ricin system, *reaper* expressing cells are efficiently cleared away by macrophages, facilitating the monitoring of cell ablation. This chapter describes the use of the *GAL4/UAS-reaper* system to specifically ablate oenocytes and thus identify their developmental role.

As a first step, three different *UAS-rpr* responder lines were tested for their efficiency in oenocyte ablation. I utilised two lines carrying one insert of the *UAS-rpr.C* transgene (*UAS-rpr[5823]* and *UAS-rpr[5824]*) as well as one line carrying 5 copies recombined into the same chromosome (*UAS-rpr[UR5S]*). The three responder lines were crossed individually to one GAL4 enhancer trap expressed in oenocytes from embryonic stage 13 onwards (*sna-GAL4,* see Section 4.3.6). As a means of easily assaying ablation frequency, this system was combined with a *svp-lacZ* reporter gene that labels the oenocytes (Elstob, Brodu et al. 2001). First instar larvae (L1) of

the control and experimental genotypes were dissected and their pelts stained for ß-galactosidase activity. Pelts from controls of the genotype *sna-GAL4; svp-lacZ* showed the normal number of oenocytes: 84-112 per larva (Figures 3.1A and 3.1B). In contrast, larvae additionally carrying the *UAS-rpr[5824]* transgene showed a substantial reduction in the number of oenocytes: 3-9 per larva (Figures 3.1C and 3.1D). Furthermore, larvae carrying either of the other two responders, *UR5S* or *UAS-rpr[5823]*, displayed complete ablation of all oenocytes (Figures 3.1E to 3.1H).

To pinpoint the time of onset and cessation of oenocyte-induced apoptosis, embryos of varying ages were immunostained with anti-ßgal. Control embryos, (*sna-GAL4; svp-lacZ*) showed persistent expression of the *lacZ* reporter until stage 17 (Figures 3.2A and 3.2B). However, experimental embryos (*UAS-rpr[5824]; sna-GAL4; svp-lacZ*) first showed characteristic signs of apoptosis, such as a reduction in the size of nuclei, together with a decrease in the intensity of *svp-lacZ* expression, from stage 15 onwards (Figures 3.2C and 3.2D). In many cases, dying oenocytes displayed defective migration from the dorsal ectoderm to a more lateral destination, adjacent to the chordotonal organ cluster Lch5 (arrowheads on Figures 3.2D and 3.2F). *UAS-rpr[5823]* and *UAS-rpr[UR5S]* gave a similar time of onset of apoptosis and in both cases the overall number of dying oenocytes was greater than with *UAS-rpr[5824]* such that by stage 17 nearly all traces of the oenocytes were missing (Figures 3.2E and 3.2F, and data not shown). However, a few nuclei remained but these were abnormally condensed and, presumably being committed to apoptosis, would be unable to perform their normal activities. All subsequent investigations were performed using one of the two strong *UAS-rpr* lines, *UAS-rpr[5823]*. This insertion was chosen as, despite the fact that there was no difference in the results using this or *UR5S*, it is homozygous viable, thus facilitating phenotypic analysis. Together, these results demonstrate that using a driver expressed in oenocytes in the embryo, the *GAL4/UAS-rpr* system can be used effectively to eliminate the complete set of oenocytes by larval hatching.

3.2 Oenocyte Ablation Does Not Affect Two Neighbouring Cell-Types But Is Larval Lethal

Oenocytes contain a high density of smooth and rough ER, resembling the structure of vertebrate secretory cells (Koller 1928; Wigglesworth 1933). This raises the possibility that they could play a secretory role, either at a paracrine or an organismal

Figure 3.1 Establishment of the *GAL4/UAS-reaper* System for Oenocyte Ablation

Dissected pelts of X-gal stained L1 larvae are shown with anterior to the left.

The *sna-GAL4* driver was crossed to three different *UAS* responder lines to assess the degree of ablation in L1 larvae. A *svp-lacZ* transgene was used to label oenocytes in all cases.

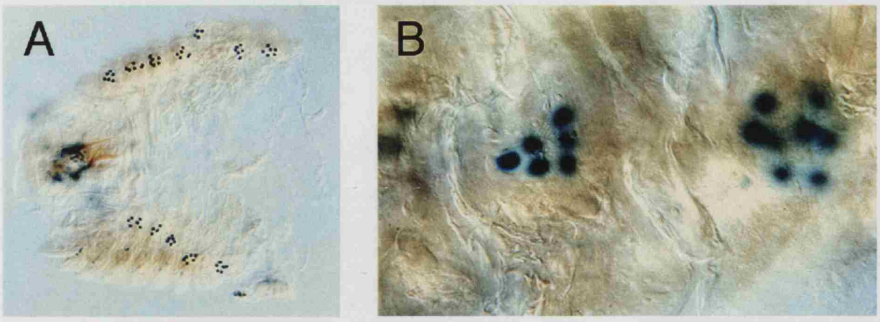
(A-B) An L1 larva without *UAS-rpr* displays the normal number of oenocytes, with each lateral clusters containing an average of 6 cells.

(C-D) *UAS-rpr[5824]* is relatively weak as seen in a larva with 9 residual oenocytes.

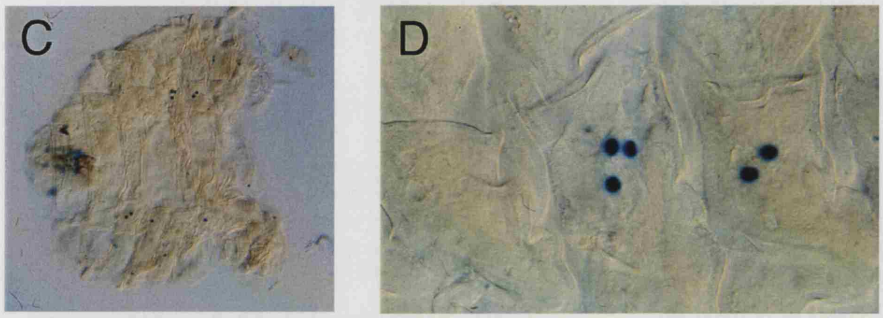
(E-H) *UAS-rpr[UR5S]* (E-F) or *UAS-rpr[5823]* (G-H) produce a complete deletion of oenocytes.

(Inset in H) Lateral chordotonal organs (arrowhead) of oenocyte-less larva retain a normal morphology.

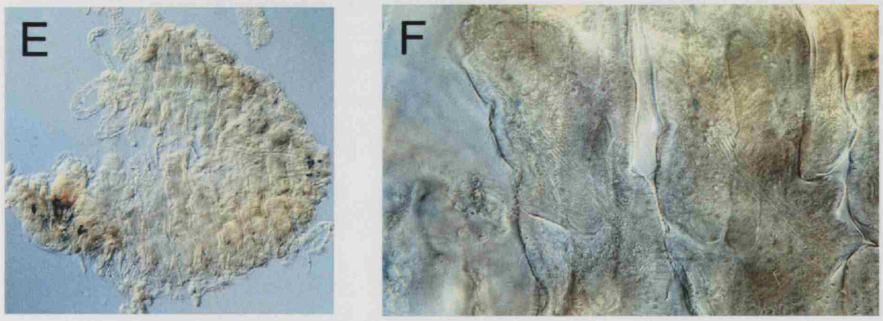
sna-GAL4; svp-lacZ



sna-GAL4; svp-lacZ x UAS-rpr[5824]



sna-GAL4; svp-lacZ x UAS-rpr [UR5S]



sna-GAL4; svp-lacZ x UAS-rpr[5823]

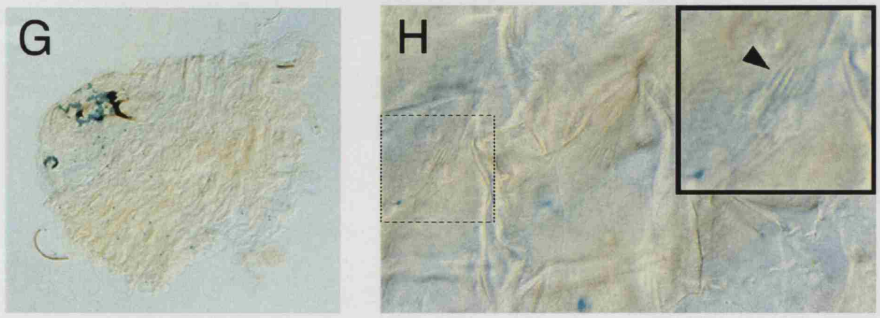


Figure 3.2 Loss of Oenocytes Does Not Affect Lateral Chordotonal Organ Morphology

In this and all subsequent figures in which embryos are shown, anterior is to the left and dorsal up, unless otherwise stated.

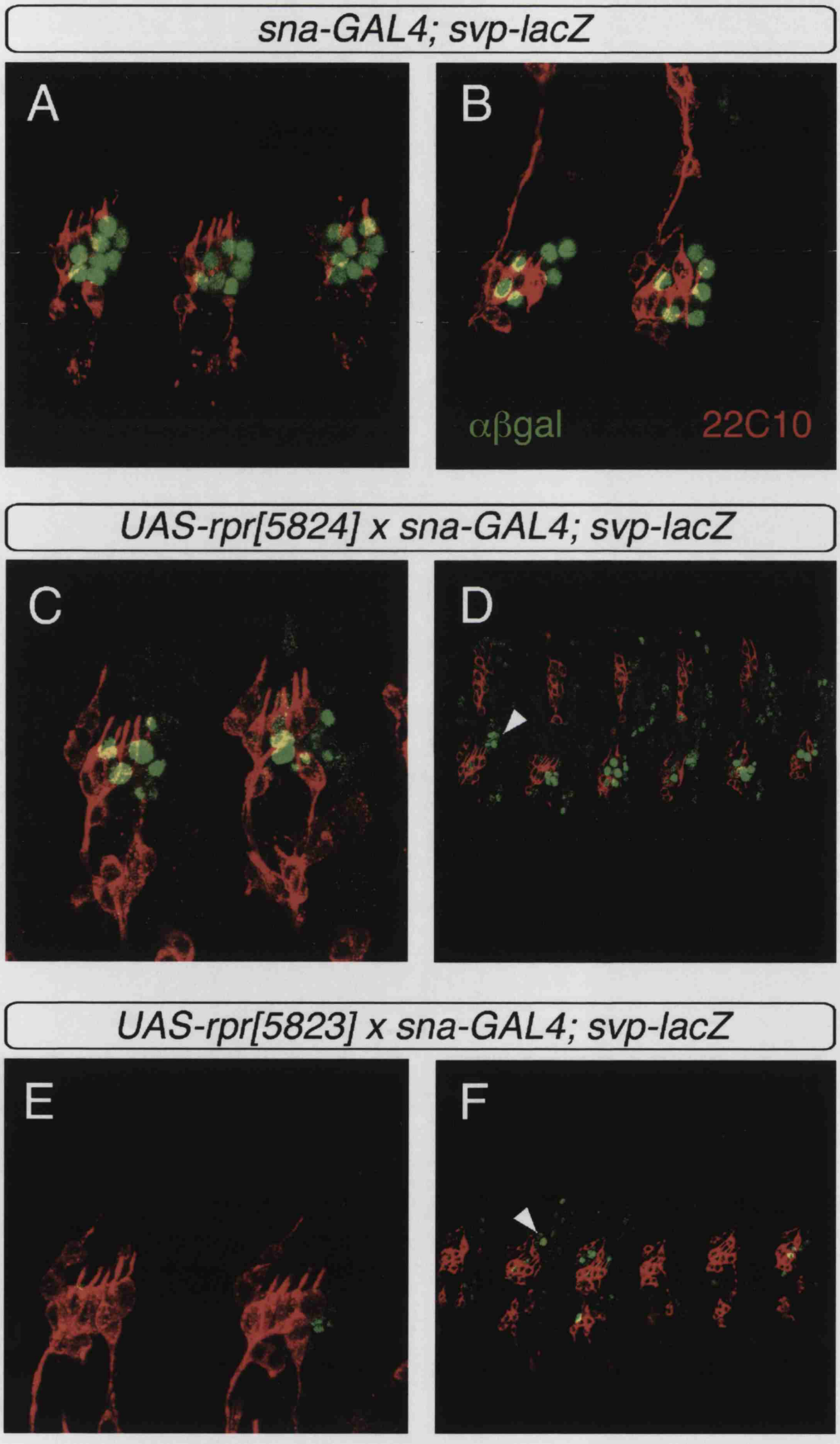
All embryos are immunolabeled with anti-ß-gal (green) and with antibody 22C10 (red).

(A-B) Stage 15 (A) and stage 17 (B) wild-type embryos showing normal arrangement of oenocytes and lateral chordotonal organs.

(C-D) Stage 15 (C) and stage 17 (D) embryos overexpressing the weak *UAS-rpr[5824]* responder line. Oenocyte clusters are mislocalised (arrowhead in D) with respect to lateral chordotonal organs and show reduced numbers and abnormal morphology.

(E-F) Stage 16 embryo (E) and a stage 17 embryo (F) overexpressing the strong *UAS-rpr[5823]*. Oenocyte misplacements (arrowhead in F) and reductions in number occur at a high frequency.

In all cases, lateral chordotonal organ morphology and position is unaffected.


sna-GAL4; svp-lacZ
A
B
αβgal
22C10
UAS-rpr[5824] x sna-GAL4; svp-lacZ
C
D
UAS-rpr[5823] x sna-GAL4; svp-lacZ
E
F

level. Two adjacent tissues have been linked previously with the oenocytes (see below): The lateral chordotonal organs and the tracheal system. Therefore, using appropriate markers, the morphology of these cell-types was studied in animals lacking oenocytes.

3.2.1. Loss of oenocytes does not affect lateral chordotonal organ morphology

The lateral chordotonal organ cluster Lch5 is closely associated with oenocytes. Not only are they juxtaposed in their final positions but the induction of oenocytes is dependent upon an adjacent Lch5 precursor cell (Section 1.7.2). First, I tested the hypothesis that oenocytes might control later stages of chordotonal organ precursor migration and/or differentiation. However, when Lch5 morphology and migration were monitored in oenocyte-deficient embryos, no evidence of mispositioning or defects in Lch5 morphology was evident (Figure 3.2A to 3.2F). Furthermore, examination of L1 pelts using DIC light microscopy, revealed no morphological abnormality of the Lch5 (Inset of Figure 3.1H). These results suggest that oenocytes do not play a role in Lch5 migration and differentiation in the late embryo. However, they do not exclude a requirement for oenocytes in Lch5 formation prior to embryonic stage 15, the time at which oenocyte apoptosis commences with *sna-GAL4*. Nor do they address whether the oenocytes affect the function of these organs as proprioceptive sensors.

3.2.2. A Developmental Switch in Pericardin Expression from Oenocytes to Chordotonal Organs

To assess the requirement of oenocytes for the late differentiation of chordotonal organs, Pericardin expression was analysed in oenocyte-less L1 larvae. Pericardin (Prc), a *Drosophila* type IV collagen alpha-chain, has been proposed to be involved in the coordinated migration of dorsal ectoderm and heart cells (Chartier, Zaffran et al. 2002). In addition to its expression in pericardial cells, it was seen in the ring gland and the oenocytes. It has been proposed that Prc is a component of the extracellular matrix. However, using Prc immunocytochemistry and confocal analysis, I noticed that most expression is not extracellular, but appears to localise as a punctuate pattern of cellular organelles within the oenocytes (Figures 3.3A and 3.3B). In addition, a striking loss of oenocyte expression, concomitant with the onset of expression in all laterally located chordotonal organs, was observed immediately after hatching

Figure 3.3 Embryo-to-larval Switch in Pericardin Expression from Oenocytes to Chordotonal Organs

All specimens are immunolabelled with anti-ß-gal (green) and Pericardin (red).

(A) Ventro-lateral view of a stage 17 control embryo showing Pericardin expression in oenocytes and dorsal bipolar neurons (dbp, arrowhead). *svp-lacZ* expression is seen in oenocytes and CNS.

(B) Single confocal section of the A5 oenocyte cluster of the embryo in (A) showing punctuate intracellular expression of Pericardin.

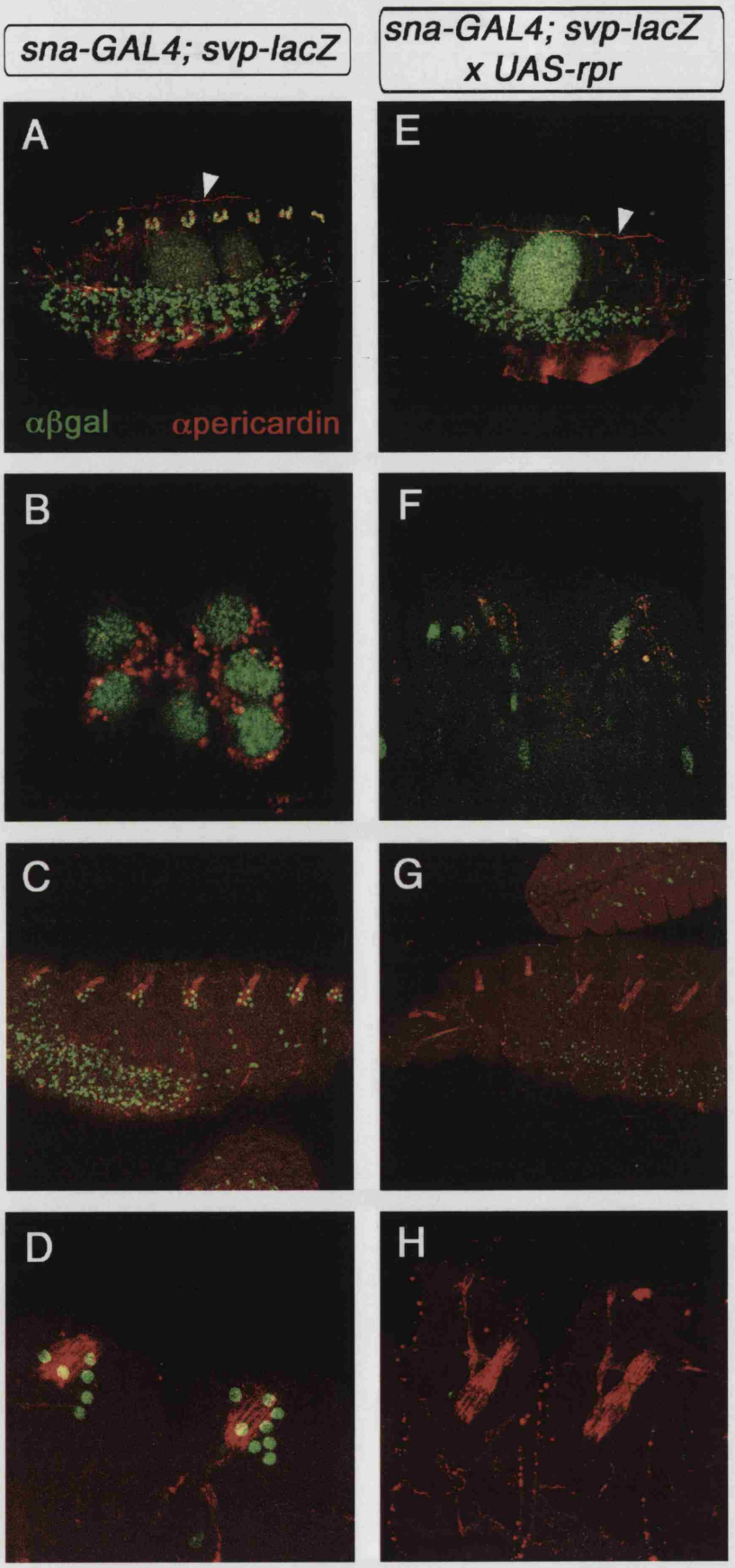
(C) An early L1 larva showing Pericardin expression in lateral chordotonal organs.

(D) Magnification of oenocyte clusters of same larva as in (C) showing loss of Pericardin expression in oenocytes but a new site of expression in chordotonal organs.

(E) Stage 17 oenocyte-less embryo showing residual Pericardin expression in dbp.

(F) Magnification of two segments of the embryo in (E), showing weak Pericardin expression in residual dying oenocytes.

(G-H) An early L1 oenocyte-less larvae showing that Pericardin expression in chordotonal organs remains unaffected.


sna-GAL4; svp-lacZ
sna-GAL4; svp-lacZ x UAS-rpr
A
αβgal
αpericardin
E
B
F
C
G
D
H

(Figures 3.3C and 3.3D). In contrast to its prior punctate expression within oenocytes, Prc seems to be localized to the cell membrane or extracellular matrix surrounding these lateral sensory organs. These observations raised the intriguing possibility that the sharp switch of Prc expression from oenocytes to chordotonal organs reflected the secretion of Prc from oenocytes and its subsequent accumulation around chordotonal organs. To test this hypothesis, the *GAL4/UAS-rpr* system was used and Prc expression observed in late embryos and early L1. Embryos lacking oenocytes showed a complete loss of Prc embryonic expression from the position where these cells should be located (Figures 3.3E and 3.3F). However, Prc larval expression in chordotonal organs remained unaffected (Figures 3.3G and 3.3H). The role of Prc and the reason for its differential subcellular localization in these different cell-types has still to be explored. However, these data do show that Prc embryonic expression in oenocytes is not required for subsequent larval expression of Prc in chordotonal organs.

3.2.3. Loss of oenocytes does not affect tracheal branching

During development, the tracheal system is patterned by positional information that specifies different tracheal cell identities (reviewed by (Uv, Cantera et al. 2003). Simultaneously, diverse local guidance cues direct the migration of tracheal cells to specific body regions (reviewed by (Uv, Cantera et al. 2003). Oenocytes are closely associated with fine tracheal branches or tracheoles, arising from the lateral trunk (Landois 1865; Wheeler 1892). In fact, for this reason, it has was suggested that they are involved in oxygen uptake. Using a GFP reporter regulated by multiple binding sites for the mammalian Hypoxia Inducible Factor 1 α/ß (HIF-1α/ß), Johnson and Krasnow have observed that when larvae are placed under mild hypoxic conditions, they initially activate GFP expression in the tracheal system and oenocytes (Johnson and Krasnow 2001); and personal communication). One possible explanation for this observation is that oenocytes are acting as an O2 sensor to prepare the rest of the animal to respond to hypoxia. Alternatively, oenocytes may possess a higher than average metabolic rate, thereby undergoing oxygen deficit earlier than other tissues. The observation that hypoxia induces the formation of fine larval tracheal branches *de novo,* using signals normally employed earlier during embryonic development (Jarecki, Johnson et al. 1999), raised the possibility that oenocytes might provide signals for normal tracheole formation during development. To test this hypothesis,

immunolabelling experiments were carried out in oenocyte-deficient embryos using the *svp-lacZ* marker and 2A12, an antibody that recognizes an unknown tracheal epitope. However, no defects in the normal tracheal branching patterns were seen (Figures 3.4A to 3.4D), indicating that tracheal formation is not dependent upon the presence of oenocytes in the late embryo.

3.2.4. Effect of ablating the oenocytes at the organismal level

The results presented thus far in this section suggest that oenocytes are not required for the normal development of adjacent tissues, such as tracheal cells and chordotonal organs. To investigate the possibility that the oenocytes could nevertheless have an essential role at the level of the whole organism, I again used the *GAL4/UAS-rpr* system with three different GAL4 enhancer traps that I previously identified (Section 4.3). Larvae carrying *sna-GAL4, Hnf4-GAL4* or *EcR-A-GAL4,* together with the *UAS-rpr[5823]* responder line were analysed for their lethal phase of development. With all three drivers, no viable adult flies were obtained (Table 3.1 and Figure 3.5). As expected for a driver expressed in the ring gland, *sna-GAL4* produced larvae that were mostly arrested during L1. *Hnf-4-GAL4* gave a polyphasic lethal phenotype, with individuals dying throughout development. The few escapers that reached the adult stage all died during the first few days. The use of *EcR-A-GAL4* resulted in a lethal phenotype at least during pupal stage, and possibly during larval stages, however, the precise stages at which death occurred were not characterised in detail. All three drivers are expressed in oenocytes within different time windows and importantly, they are also expressed in various other tissues (Table 3.1). Presumably, these two differences may account for the non-identical lethal phases of the drivers. In summary, even though these data suggest that oenocytes may play an essential role during development, their function could not be disentangled from that of other tissues deleted by the three ablation systems.

3.3 Construction of oenocyte-specific GAL4 drivers

To dissect out the function of oenocytes cleanly, a more selective ablation approach was required. Towards this aim, more specific oenocyte-GAL4 drivers were engineered. To select the most specific enhancer elements for cloning into a *GAL4* construct, the expression pattern of three highly-restricted *lacZ* lines expressed in oenocytes was explored in depth.

Figure 3.4 Loss of Oenocytes Does Not Affect Tracheal Branching

Embryos are immunolabelled with anti-ß-gal (green) and with tracheal antibody 2A12 (red).

(A-B) A stage 16 control embryo showing a normal tracheal branching pattern.

(C-D) Tracheal branch formation is unaltered in stage 16 oenocyte-less embryos.

Arrowheads in (A) and (C) indicate the section of the lateral trunk to which the oenocytes are connected.

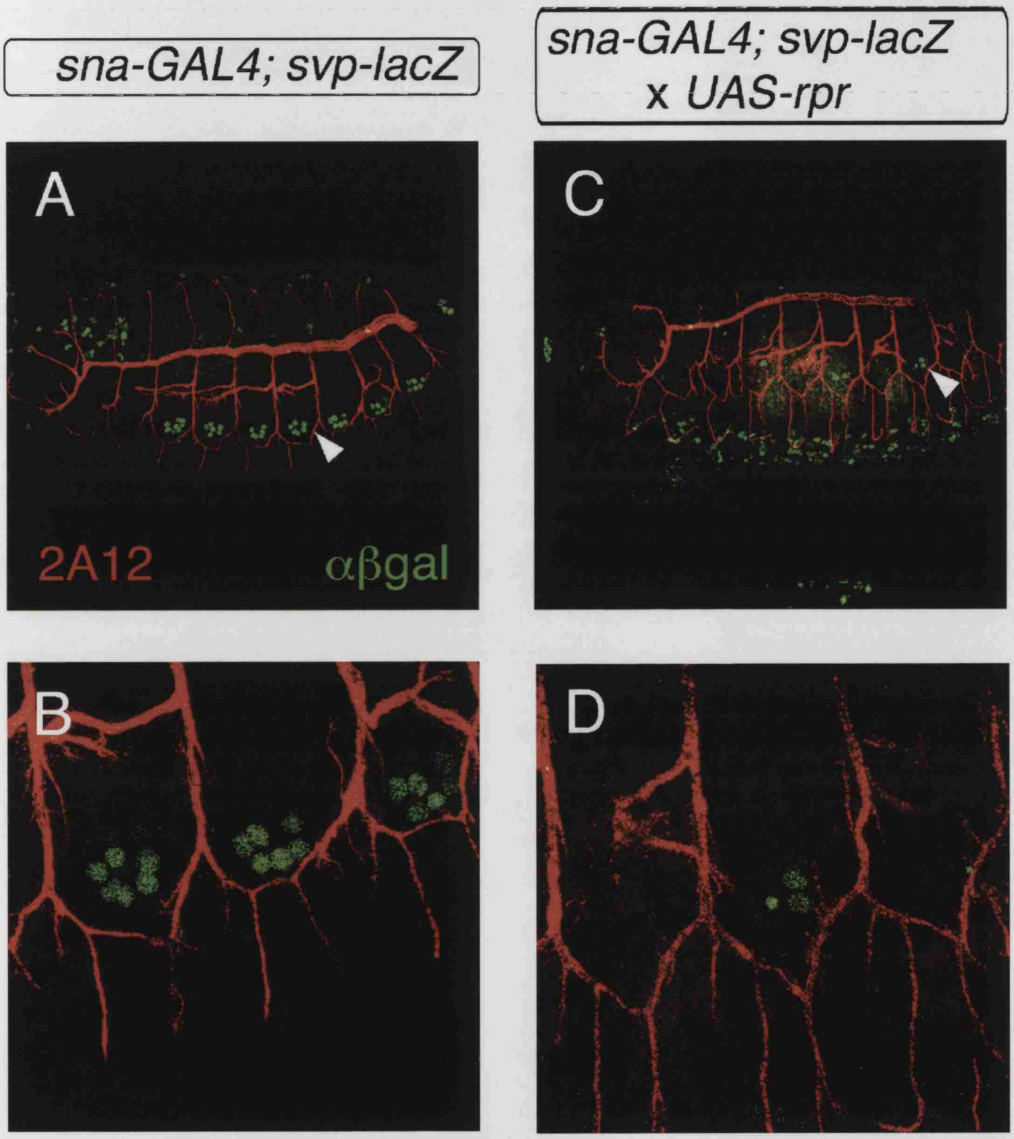

sna-GAL4; svp-lacZ
sna-GAL4; svp-lacZ
x UAS-rpr
A
C
2A12
αβgal
B
D

Table 3.1 Lethal Phase of Three Oenocyte Drivers with *UAS-reaper*

The three drivers indicated here were identified in an enhancer trap screen (Section 4). Larval expression was assessed at 96hrs post hatching.

Lethal phases were determined in a similar way to Fig. 3.5 but L1, L2, L3 stages were not distinguished.

SG refers to Salivary Glands.

+/– indicates presence or absence of *UAS-nlacZ* expression in oenocytes in embryos or larvae.

Driver	Oenocyte Expression		Expression in other tissues		Lethal phase
	Embryo	L3 Larva	Embryo	L3 Larva	
sna-GAL4	+	+	SG, ring gland, few epidermal cells	SG, ring gland, CNS, gut	Larval
Hnf4-GAL4	–	+	SG, few cells in gut	SG, epidermis, hindgut, fat body, CNS, eye discs, proventriculus	Larval and Pupal
EcR-A-GAL4	+	–	SG, posterior hindgut, apodemes	SG, fat body, epidermis, wing disc, esophagus	Larval and/or Pupal

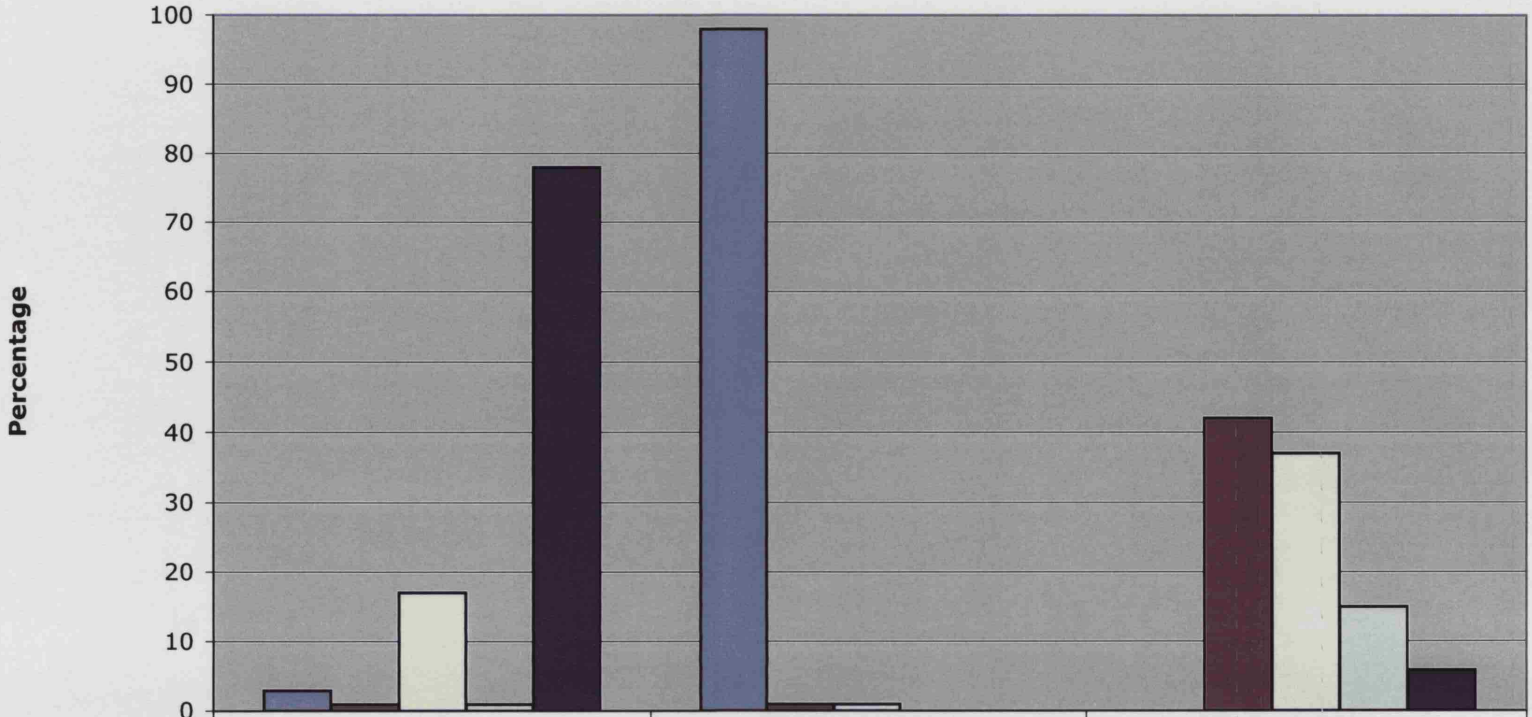
Figure 3.5 Lethal Phases of Two Drivers Expressed in Oenocytes Combined with *UAS-reaper*

Graphs show the percentage of larvae dying during L1, L2, L3 or pupal stages, using the 2 different driver lines indicated.

For all lethality profiles, L3 refers to larvae dying either during the L2/L3 moult or as L3 and live adults refers to the percentage of flies that eclose in each case.

The experiments described in this Figure were done in parallel with those described in Figure 3.11 and used the same *yw* control.

Lethal phases of two drivers expressed in oenocytes combined with *UAS-rpr*



	yw	*sna-GAL4[P0206]*	*Hnf4-GAL4[OK72]*
L1	*3*	*98*	*0*
L2	*1*	*1*	*42*
L3	*17*	*1*	*37*
Pupa	*1*	*0*	*15*
Live adults	*78*	*0*	*6*

3.3.1. *ng1-lacZ*

An 83bp genomic fragment of the *(new glue 1) ng1* regulatory region, containing an ecdysone responsive element directing high levels of expression in salivary glands (Crispi, Giordano et al. 2001), was also previously shown to confer reporter gene expression in oenocytes (Crispi, Giordano et al. 1998). Detailed analysis of the expression in this reporter line (*ng1-lacZ[IPR]*) showed that it began in oenocytes at embryonic stage 12 and was maintained throughout embryogenesis (data not shown). However, I observed reporter expression in two clusters of cells located at the posterior tip of the embryo, adjacent to the posterior spiracles (data not shown and Appendix Figure 1F). In addition, larval expression in the CNS, with a pattern suggestive of glial or tracheal identity, was also noted (data not shown). Based on the non-oenocyte expression domains and the high probability that this small DNA fragment would be very sensitive to position effects (Crispi, Giordano et al. 1998; Crispi, Giordano et al. 2001), *ng1* was ruled out as a suitable oenocyte-specific enhancer element.

3.3.2. *svp3kb-lacZ* and derived GAL4 lines

The next element analysed was a *lacZ* construct containing a 3kb region of the *svp* promoter reported to be expressed in oenocytes (Schulz RA, unpublished and 3.6A). A detailed study of this reporter construct revealed expression in oenocytes (Figure 3.7A), beginning from embryonic stage 13. Other tissues that showed embryonic expression were the ring gland, esophageal cells, salivary glands and some unidentified cells at the anterior tip of the embryo (Figure 3.6B). In L3, expression was maintained in the ring gland, esophagus and oenocytes (Figures 3.7C and 3.7D). In addition, expression in the epidermis, spiracular glands and the salivary glands was detected (Figure 3.7D and Figure 3.6B). No expression in the CNS was detected (Figure 3.7C). The promoter fragment analysed was 3kb in size, raising the possibility that an individual module contained within it might regulate expression in an oenocyte-specific manner. To attempt to separate enhancer elements, the *svp3kb* element was divided into 1.7kb and 1.3kb fragments and each was cloned into the *pWHS-GAL4* vector (C. Cenci and A. Gould, unpublished, see Materials and Methods), so that several driver lines could be established. To test the specificity of expression of each construct, they were crossed to a *UAS-lacZ* responder line.

Figure 3.6 The *svp3kb* Enhancer Reporter Analysis

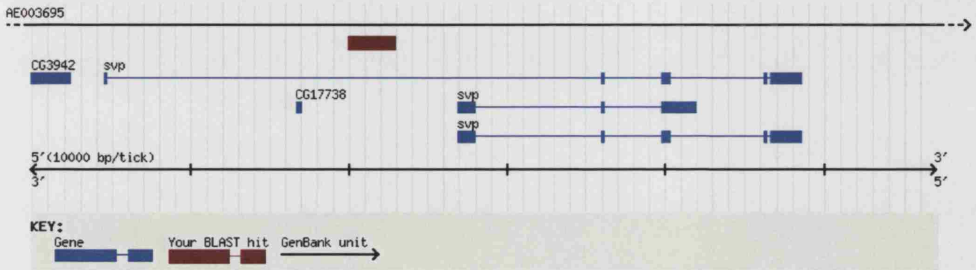
(A) Diagram of the 3kb enhancer region (red) of the *seven-up (svp)* locus indicating its position with respect to the three *svp* isoforms (blue).

(B) Table comparing the expression domains of the *svp3kb-lacZ* transgene and the different GAL4 drivers generated.

* Embryonic expression was consistently restricted to oenocytes, ring gland and anterior tip.

Oenos refers to oenocytes; Spi G: spiracular glands; Epi: epidermis; FB: Fat body; No. lines: number of independent insertions analysed for each construct; ND: not determined.

A



B

	Embryo*	Larva							No. Lines
		Oenos	RG	Spi. G	Epi	FB	Gut	Trachea	
lacZ — svp3kb-lacZ	+	+	+	+	+	–	–	–	2
GAL4 — svp1.7kb-GAL4	–	–	–	ND	–	+	+	+	2
GAL4 — svp1.3kb-GAL4	+	–	+	+	+	–	–	–	2
GAL4 — svp0.5kb-GAL4	ND	–	+	–	–	–	+	+	4
GAL4 — svp0.8kb-GAL4	–	–	–	–	–	–	–	–	1

Figure 3.7 *svp3kb-lacZ* and *svp1.3kb-GAL4* Are Expressed in Oenocytes

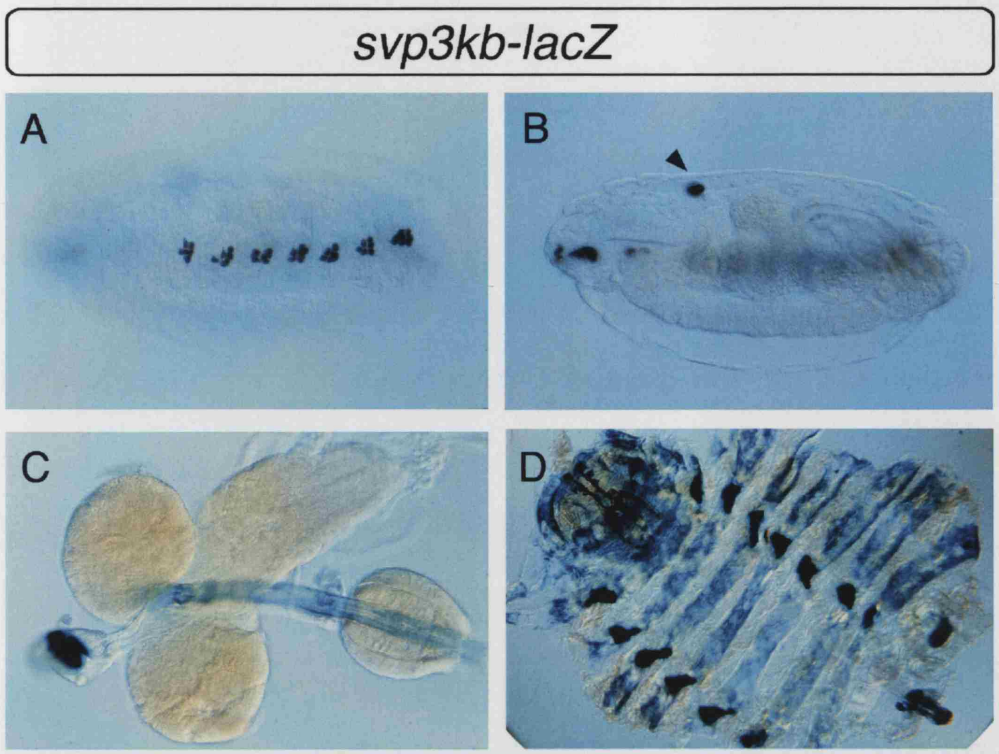
Embryos are immunolabelled against ß-gal. X-gal staining was used on dissected larvae.

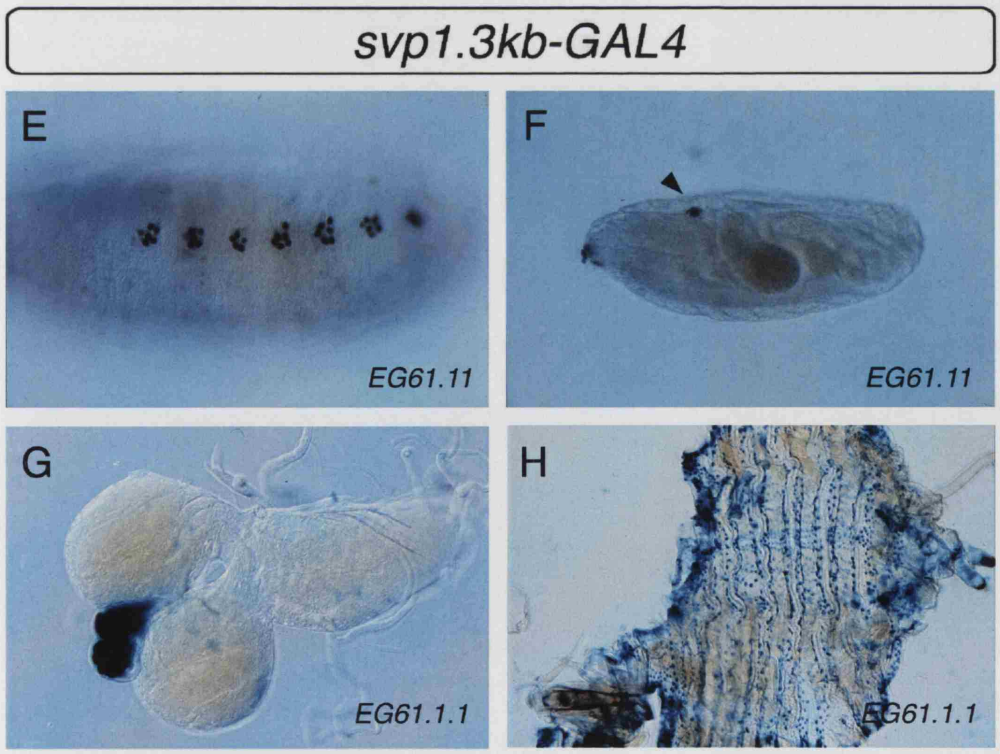
(A-D) *svp3kb-lacZ* transgene labels oenocytes and some other embryonic and larval tissues.

(A-B) External (A) and internal (B) focal planes of a stage 17 embryo showing restricted *lacZ* expression in oenocytes (A), ring gland (arrowhead in B), esophageal cells and a cluster of cells in the anterior tip.

(C-D) *lacZ* expression in L3 larvae in L3 larvae in the ring gland (C), esophagus (C) and oenocytes (D) of an L3 larva. Additional larval sites of expression are the epidermis (D) and spiracular glands (D and Appendix Figure 1D).

(E-H) *svp1.3kb-GAL4* recapitulates most of the *svp-3kb* expression pattern. (E-F) A *svp1.3kb-GAL4* stage 17 embryo recapitulates the embryonic expression of the *svp3kb* enhancer oenocytes (E), ring gland (arrowhead in F) and anterior tip (F). (G-H) An L3 *svp3kb-GAL4* larva shows expression in the ring gland (G), epidermis (H) and spiracular glands (H). There is no expression in L3 oenocytes.


svp3kb-lacZ
A
B
C
D


svp1.3kb-GAL4
E
EG61.11
F
EG61.11
G
EG61.1.1
H
EG61.1.1

Analysis of two independent *svp1.7kb-GAL4* insertions revealed that the embryonic expression seen with the *3kb-lacZ* construct was completely absent, as was the larval expression in oenocytes, ring gland and epidermis (Figure 3.6B). In contrast, one insertion of the *svp1.3kb-GAL4* construct showed a very similar expression pattern to the *svp3kb-lacZ* progenitor construct. The oenocytes, the ring gland and some cells at the anterior tip expressed *UAS-lacZ* during embryonic stages (Figures 3.7E and 3.7F). Expression in the oenocytes begins at late stage 12 to early stage 13 and is subsequently maintained throughout embryogenesis. During L3, expression was detected in the ring gland, epidermis, spiracular glands and salivary glands (Figures 3.7G and 3.7H and Figure 3.6B). Surprisingly, *svp1.3kb-GAL4* differed from *svp3kb-lacZ* in that expression in oenocytes was only observed during embryonic stages and not in L3 (compare Figure 3.7D to Figure 3.7H).

In order to further refine the *GAL4* expression pattern, the 1.3kb *svp* enhancer was further subdivided into 0.5kb and 0.8kb fragments and cloned into *pWHS-GAL4*. Analysis of this second round of transformant fly lines revealed no expression in one insertion of the *svp0.8kb-GAL4* construct in embryos and only weak larval staining in the salivary glands, the gonads and some cells in the brain lobes (Figure 3.6B and data not shown). On the other hand, larvae from 4 independent lines of *svp0.5kb-GAL4* showed consistent expression in salivary glands, ring gland, tracheal cells and a subset of intestinal cells (Figure 3.6B). The embryonic expression pattern of the *svp0.5kb-GAL4* construct remains to be determined.

Together, the transgenic analysis of the *svp* promoter region suggests that regulatory elements required for embryonic oenocyte expression lie within a 1.3kb region, located 3918bp and 3960bp upstream of *svp* transcripts *A* and *B*, respectively (Figure 3.6A). In addition to embryonic oenocyte expression, the 1.7kb fragment may contain elements cooperating with the 1.3kb region to maintain expression in the oenocytes of L3. Within the 1.3kb region, elements contained in a 0.8kb fragment are required for larval repression in the gut and tracheal cells, while elements contained in a 0.5kb fragment are sufficient to drive expression in the ring gland (Figure 3.6B). In summary, an element that solely drives expression in the oenocytes was not isolated in these experiments, but further analysis of the 0.5kb region in the future may still identify one. Nevertheless, the *svp* promoter analysis did lead to the construction of a useful *GAL4* driver expressed in oenocytes, ring gland and some other tissues.

3.3.3. *BO32-lacZ* and derived GAL4 lines

A 7.6kb enhancer of the *sal* complex, named the *BO* fragment, has been reported to be specifically expressed in oenocytes during embryogenesis, later being expressed in the wing, haltere and eye imaginal discs of the larva (Barrio, de Celis et al. 1999). My embryonic analysis of one *BO32-lacZ* line confirms that the *BO* element is oenocyte-specific (Figures 3.8A to 3.8D), with expression beginning at embryonic stage 14. Interestingly, a low level of expression was also noted in the same cluster of cells located at the posterior tip of the embryo as described with the *ngl-lacZ* construct (Arrowhead in Appendix Figure 1B). Expression during L3 revealed few sites of expression in addition to the imaginal discs. No expression was seen in the CNS (Figure 3.8F), the gut, trachae or fat body (Figure 3.8F and Figure 3.8I). Importantly, no expression was detected in the epidermis or the ring gland (Figures 3.8E and arrowhead in 3.8H, respectively). Thus, the detailed expression analysis of the *BO* enhancer, indicates that it is the most suitable of the candidates tested for constructing a *GAL4* driver for expression in selective oenocyte ablation experiments.

To construct the oenocyte-specific *GAL4* driver, the intact 7.6kb *BO* element was cloned into the *pWHS-GAL4* transformation construct and 11 independent transformant lines were established. To test whether these lines reflected the activity of the *BO* element predicted from *BO-lacZ*, each line was crossed individually to *UAS-lacZ*. In agreement with the *BO-lacZ* analysis, the 11 lines consistently gave expression in the oenocytes (data not shown), starting from embryonic stage 12 through to the end of embryogenesis. In addition, unexpected larval expression in epidermis, tracheal cells and oenocytes was observed (data not shown). However, unexpected expression was also found in ventral epidermal stripes, first appearing in stage 17 embryos (data not shown). Examination of larval tissues of 9 independent insertions revealed expression in imaginal discs, gonads and salivary glands, as expected from the *BO-lacZ* construct. These data show that the *BO-GAL4(pWHSpe)* driver construct can be used as an oenocyte-specific *GAL4* driver for most of embryogenesis but starting at embryonic stage 17 and throughout larval life, its expression becomes more widespread, reducing its potential utility.

The appearance of additional expression sites in the *BO-GAL4(pWHSpe)* transgene was unlikely to be caused by positional effects, as the same additional expression sites appeared in most of the lines analysed. Furthermore, the possibility that the reported expression pattern for the *BO-lacZ* construct was itself affected by its

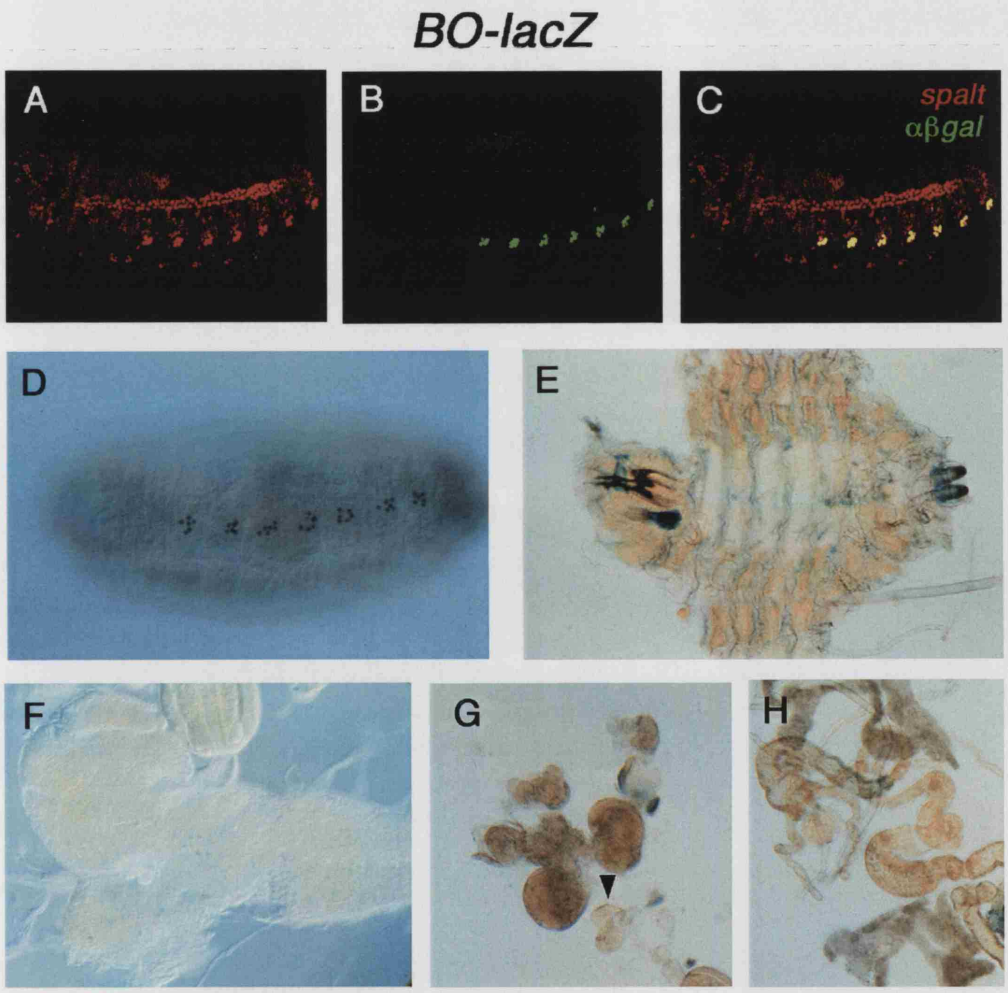
Figure 3.8 *BO-lacZ* Expression is Specific for the Oenocytes

(A-C) A stage 13 embryo immunolabeled with anti-ß-gal (green) and Spalt (red). The *BO* element from the *spalt* complex is uniquely expressed in the oenocytes but not in other *spalt* domains.

(D) A stage 17 embryo immunolabelled with anti-ß-gal shows specific expression restricted to the oenocytes.

(E-I) Dissected L3 larva shows no expression of the *BO-lacZ* transgene in epidermis (E), CNS (F), ring gland (arrowhead in G), trachea (H), fat body (H) or gut (H). However, weak expression is seen in eye disc (G) and other imaginal discs (data not shown).

Panels A-C were kindly provided by Alex Gould.


BO-lacZ
A
B
C
spalt
αβgal
D
E
F
G
H

position in the genome was also unlikely as three independent lines were analysed (Barrio, de Celis et al. 1999). An alternative explanation for the divergence in expression between *BO-lacZ* and *BO-GAL4(pWHSpe)* might be the different promoter architecture of these two transgenes. *BO-lacZ* was constructed by subcloning *BO* into the *EcoR I* restriction site of C4PLZ (Wharton and Crews 1993; Barrio, de Celis et al. 1999). In this vector, the *lacZ* transgene is fused to the P-element transposase basal promoter. Instead, the *pWHSpe* vector made use of the *hsp70* basal promoter to drive expression of *GAL4*. Based on the hypothesis that loss of specificity might be caused by inappropriate promoter architecture, I engineered a new *GAL4* vector (*pC4G4)* to mimic the way in which *BO* interacts with the basal transcriptional machinery within *BO-lacZ* (for full description of this construct refer to Materials and Methods). 24 independent lines of *BO-GAL4(pC4G4)* were established (Table 3.2). Similar larval expression domains to *BO-GAL4(pWHSpe)* insertions were observed in 19 lines, while 5 of the insertions were far more oenocyte-specific. Fortunately, two of these (*EG79.7* and *EG79.26*) showed a larval expression pattern virtually identical to that seen with *BO-lacZ*. These two lines were further characterised by studying their embryonic expression pattern in detail. *EG79.7* and *EG79.26* embryos both showed strong and specific oenocyte expression (Figure 3.9A and data not shown). This expression begins at stage 13 in a patchy manner and quickly resolves into strong expression by stage 14. Importantly, the ventral epidermal expression seen in *BO-GAL4(pWHSpe)* embryos was completely absent. L3 larvae also show moderate expression in the oenocytes (Figures 3.9B and 3.9C), with weak staining in the posterior spiracles (Figure 3.9B), eye discs (Figure 3.9D), a small subset of tracheal cells and the salivary glands (data not shown).The epidermis (Figure 3.9B), ring gland (Figure 3.9F), and other essential tissues such as the fat body, gut and Malpighian tubules (data not shown) do not express the transgene. Thus, importantly, the only non-oenocyte sites of expression of this line were in tissues known not to be essential for viable development from embryonic to pupal stages (Manning and Krasnow 1993; Jones, Kuo et al. 1998). *GAL4* expression in *EG79.7* and *EG79.26* larvae was very similar with the exception that CNS expression in developing adult optic lobes was detected with *EG79.26* only. These data show that *BO-GAL4(pC4G4)* can be used to drive expression of different reporter genes in a highly oenocyte-specific manner. In conclusion, after several rounds of constructs, the *BO-GAL4* insertions *EG79.7* and *EG79.26* appear promising for use in the oenocyte ablation experiments.

Table 3.2 Summary of 24 *BO-GAL4(pC4G4)* Larval Expression Patterns

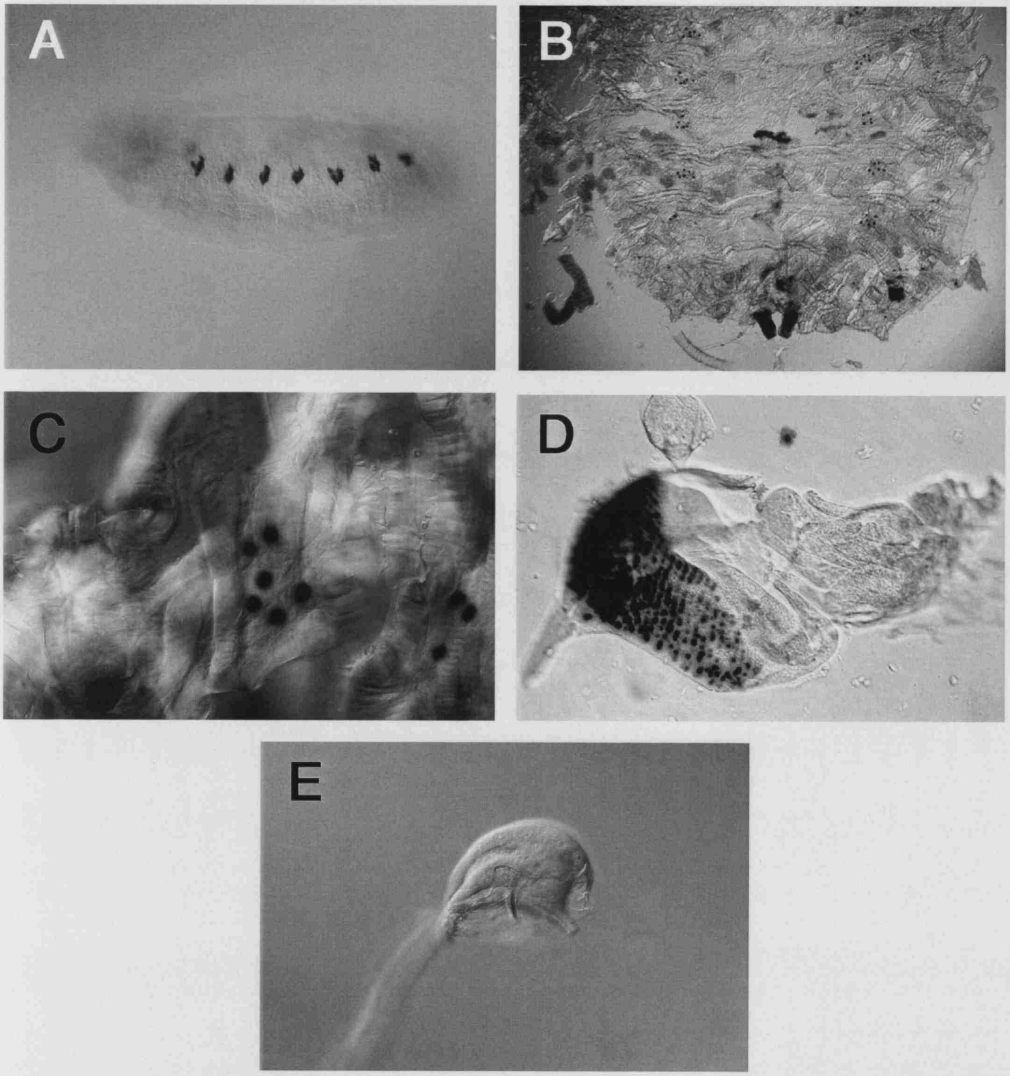
In this table + refers to strong expression in all lines; ± means weak variable expression; – refers to no expression; ND indicates not determined

Number of Independent Insertions	**Oenocytes**	Epidermis	Trachea	CNS	Ring gland
19	**ND**	+	ND	ND	ND
2 (EG79.22 & EG79.10)	+	±	+	+	—
1 (EG79.8)	+	—	+	±	ND
2 (EG79.7 & EG79.26)	+	—	±	±	—

Figure 3.9 The *BO-pC4G4[EG79.7]* Line Can Be Used as an Oenocyte Specific Driver

(A) Stage 17 embryo immunolabelled against ß-gal shows specific oenocyte staining. (B-G) Dissected L3 larva showing moderate and highly restricted transgene expression in oenocyte clusters (B and C), posterior spiracles (B), eye discs (D). Importantly, the ring gland does not express the transgene (E).

BO-pC4G4



3.4 Oenocyte Ablation is Associated with Developmental Arrest and Moulting Defects

To investigate the possibility that oenocytes have an essential role during development, the two oenocyte-specific *BO-GAL4* drivers *EG79.7* and *EG79.26* were combined with *UAS-rpr[5823]*. Embryos carrying these *GAL4/UAS* combinations were raised in parallel with control embryos (*yw* x *UAS-rpr*). In both cases, oenocytes were completely removed as seen by the disappearance of the *svp-lacZ* reporter (data not shown). As with one of the less oenocyte-specific drivers used previously, experimental animals failed to puparate. Experimental but not control larvae displayed a syndrome of phenotypes including asynchronous development, larval arrest, arrest in food intake, decreased body size, transparent cuticle, reduced size and abnormal appearance of fat body, and duplicated exoskeletal components. The majority of oenocyteless larvae die within the first 4 days of larval life, with a minority remaining arrested in the L2 and L3 stages for several weeks. In addition, larval development was highly asynchronous, with individual larvae developing at very different rates. Another observation that was not quantified was that, although L1 oenocyte-less larvae appear to have normal behaviour, L2 oenocyte-less larvae stop feeding and crawl out of the food, a behaviour normally associated with the end of L3. Importantly, the ring gland of such individuals appears to be normal as seen on an L3 larva arrested for 10 days (Figure 3.10A) or the ring gland from a similar specimen, in this case conveniently labelled with a *svp-lacZ* marker expressed in part of the ring gland and CNS (Figure 3.10B).

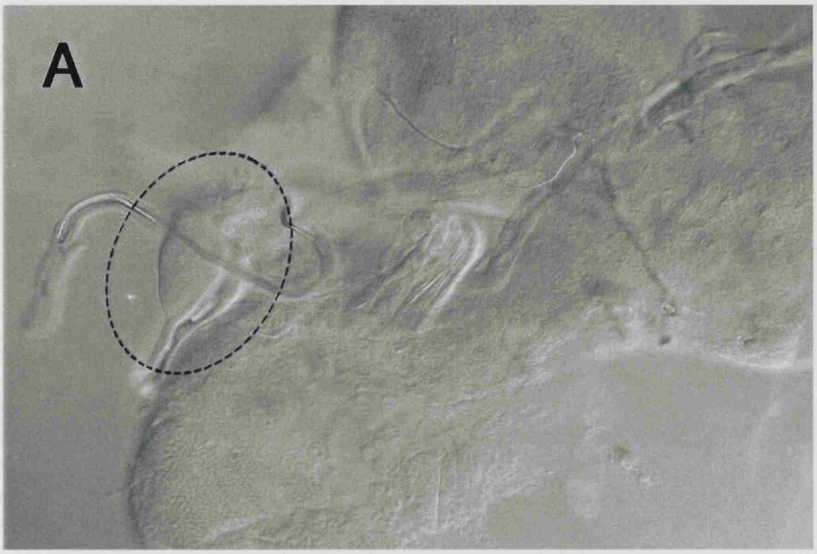
Next, a more detailed analysis of lethal phases was performed (Figure 3.11), using staging criteria based on morphological features such as the serration pattern of the mouth hooks and the structure of the anterior spiracles (Bodenstein 1950). Control larvae showed only a low level of larval lethality. In contrast, *EG79.7* gave severe polyphasic lethality with about one third of the individuals dying during L2 and the remaining two thirds dying during the L2/L3 moult or as L3. With the other driver *EG79.26* the vast majority of larvae died during the L2/L3 moult or as L3, with a few specimens dying as L2. The moderate difference in the lethality profiles of the two *BO-GAL4* drivers tested may be caused by different levels of expression of *rpr*, perhaps leading to a different time course of oenocyte ablation. However, both drivers

Figure 3.10 Oenocyte-less Larvae Have a Normal Ring Gland

(A) A 10 day oenocyte-less L3 larva with a ring gland (dotted circle) that appears morphologically normal.

(B) Detail of a 4-day oenocyte-less larva showing the ring gland, part of which is labelled by the *svp-lacZ* transgene (arrowhead).

UAS-rpr[5823] x BO-pC4G4[EG79.7]



UAS-rpr[5823];; S10/TM3
x BO-pC4G4[EG79.26]

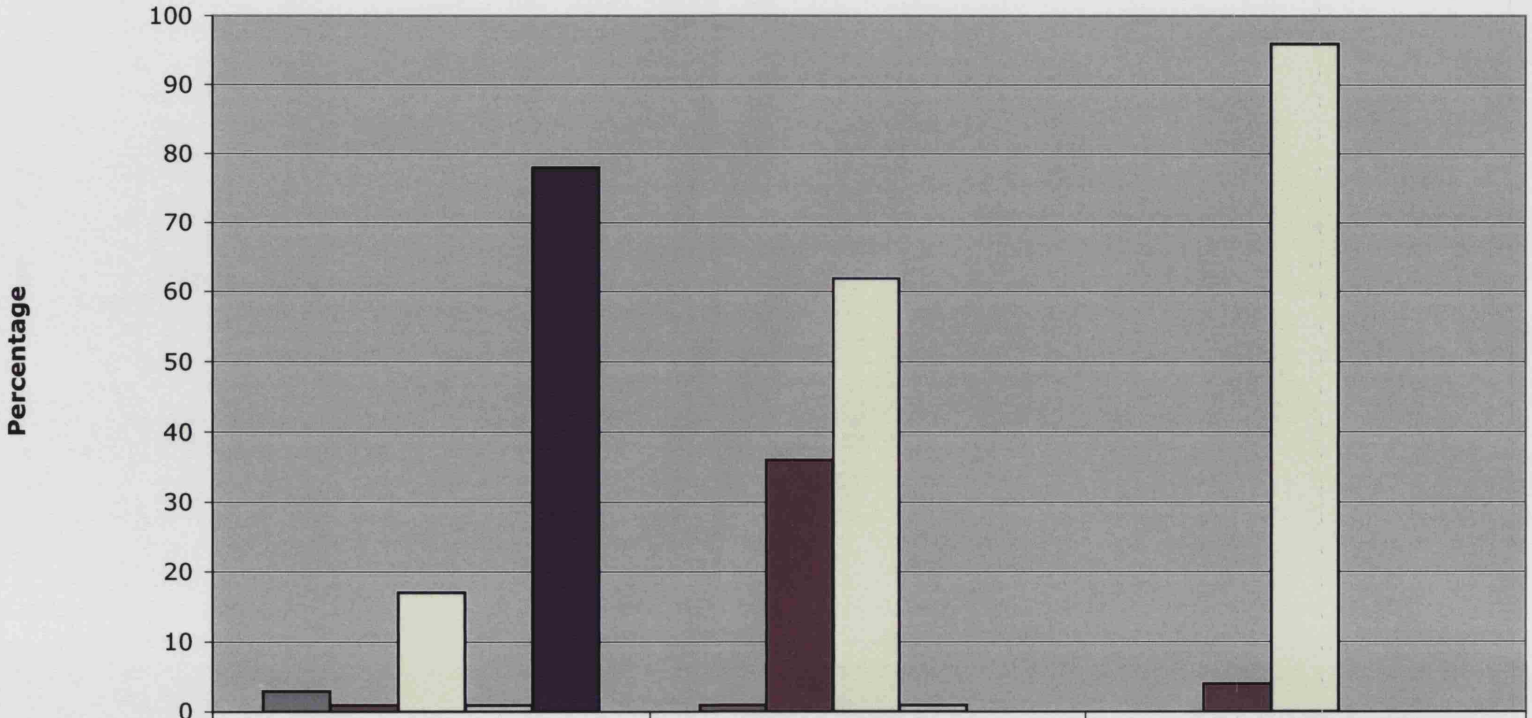
Figure 3.11 Lethal Phases of Two *BO-GAL4* Drivers Combined with *UAS-reaper*

Graphs show the percentage of larvae dying during L1, L2, L3 or pupal stages, using the 2 different driver lines indicated.

For all lethality profiles, L3 refers to larvae dying either during the L2/L3 moult or as L3 and live adults refers to the percentage of flies that eclose in each case.

The experiments described in this Figure were done in parallel with those described in Figure 3.5 and used the same *yw* control.

Lethal phases of two *BO-GAL4* drivers combined with *UAS-rpr*



	yw	*BO-GAL4[EG79.7]*	*BO-GAL4[EG79.26]*
L1	*3*	*1*	*0*
L2	*1*	*36*	*4*
L3	*17*	*62*	*96*
Pupa	*1*	*1*	*0*
Live adults	*78*	*0*	*0*

produce broadly similar phenotypes affecting developmental progression and food intake, thus suggesting that oenocytes may play a role in regulating these processes.

To characterise further the larval arrest syndrome, the morphologies of mutant and control larvae were compared side by side. Mutant larvae posessed posterior spiracles that were retracted and never protruded out of the body, as they would normally do (Figure 3.12A). Three categories of developmentally arrested larvae were observed: persistent L2, L3 with residual L2 exoskeleton, and persistent L3 that had successfully moulted. Oenocyte-less larvae vary greatly in size with many persistent L2 larvae being comparable in size to late L3 controls. Conversely, many persistent L3 larvae were found to be much smaller, with a size normally associated with L2 stages (Figure 3.12B). After several days of arrest, a fraction of these larvae showed an abnormally transparent cuticle, a slim body shape and a reduced amount of fat body (Figure 3.12B). Such fat body showed an abnormal appearance containing bigger granules than wild-type (data not shown). Mutant larvae showed several manifestations of exoskeleton duplication, varying from specimens displaying a complete duplicated exoskeleton to others that showed duplication of only some of its components, such as the mouth hooks (compare Figures 3.13A and 3.13C), anterior spiracles (Figures 3.13B and 3.13D) or tracheal branches (Figures 3.13E to 3.132G). Interestingly, the duplicated mouth hook phenotype was also observed previously when *sna-GAL4* and *Hnf4-GAL4* were used (Figures 3.14A and 3.14B). This strongly suggests that exoskeleton duplications and larval arrest are associated with the ablation of oenocytes and not some other tissue-type.

3.5 Ecdysteroids Treatment as Means of Overcoming the Oenocyte-less Phenotype

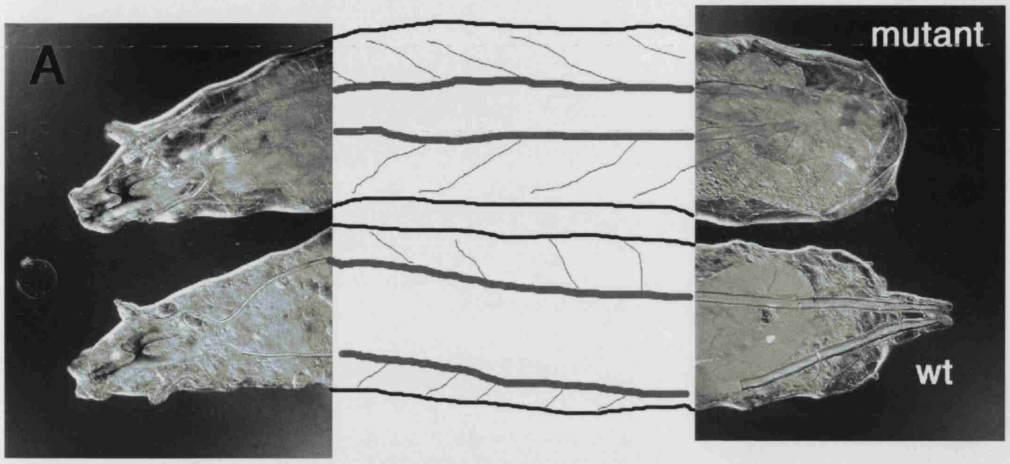
Known mutants affecting the moulting process fall into three general categories depending on whether they primarily affect ecdysone synthesis and secretion, downstream transcriptional signalling or peptide hormone processing (Section 1.6.2). Several of the phenotypes present in oenocyte-less larvae closely resemble those of known moulting mutants, in agreement with the previously hypothesis that oenocytes are involved in moulting (Section 1.7.4). There are three possible ways in which oenocyte function might influence moulting. In one scenario, the oenocytes could be required upstream of the ring gland, providing a permissive signal for the correct release of ecdysone. A prediction from this hypothesis is that such a phenotype should be rescued by providing exogenous ecdysone or 20-E. A second possibility is that the

Figure 3.12 Oenocyte Ablation Is Associated With Larval Arrest

(A) Comparison of a 4 day wild-type L3 wandering larva *(yw/UAS-rpr)* to an L3 14-day old oenocyte-less larva *(BO-pC4G4[EG79.7]>rpr)*. The oenocyte-less larva shows retracted spiracles but after 10 days delay has the normal size.

(B) Two arrested and undersized oenocyte-less larvae compared to an L3 wandering wild-type larva. The three larvae shown in this panel are in L3, based on mouth-hook and anterior spiracle morphology. Upper mutant and wild-type larvae were 4 days old. Lower mutant is 18 days old.

BO-pC4G4[EG79.7] X UAS-rpr



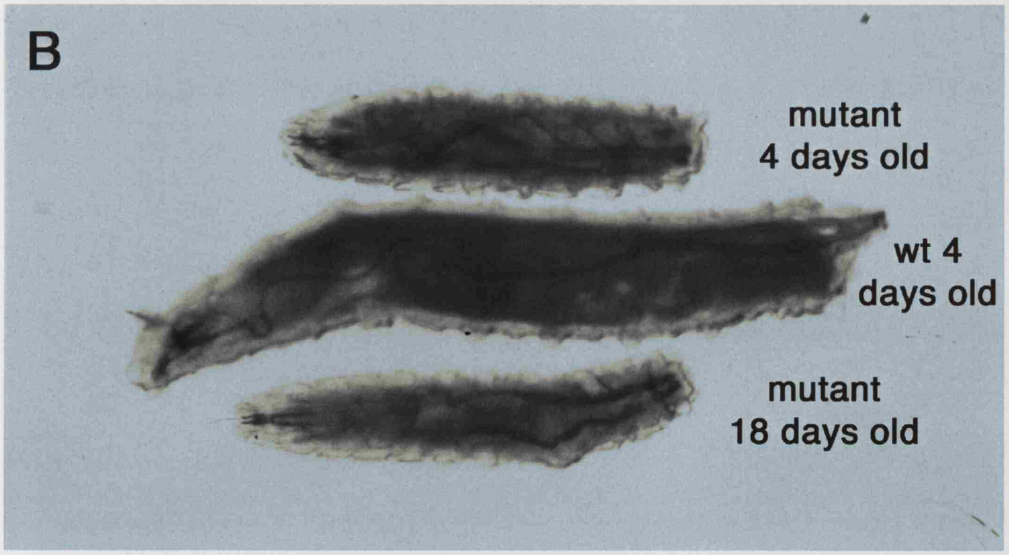


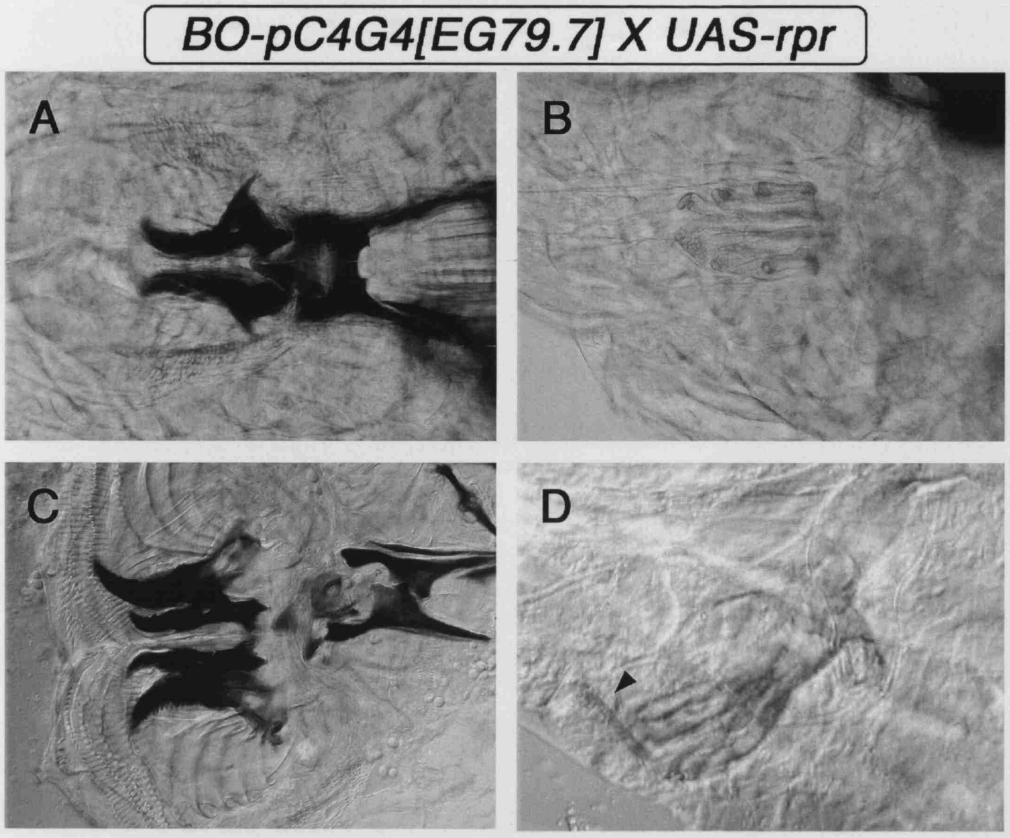
Figure 3.13 Oenocyte-less Larvae Display a Moulting Phenotype

Both the *EG79.7* (A-D) and *EG79.26* (E-G) drivers, together with *UAS-reaper* produce duplicated exoskeletal structures.

(A-B) Oenocyteless larva showing normal L3 mouth hooks (A) and L3 anterior spiracle morphology.

(C-D) Oenocyteless larva showing L2 and L3 duplicated mouth hooks (C) and duplicated anterior spiracles (D).

(E-G) Three panels showing duplicated tracheal branches of oenocyte-less larvae. Arrowheads point to residual L2 tracheal branches inside L3 branches.


BO-pC4G4[EG79.7] X UAS-rpr
A
B
C
D

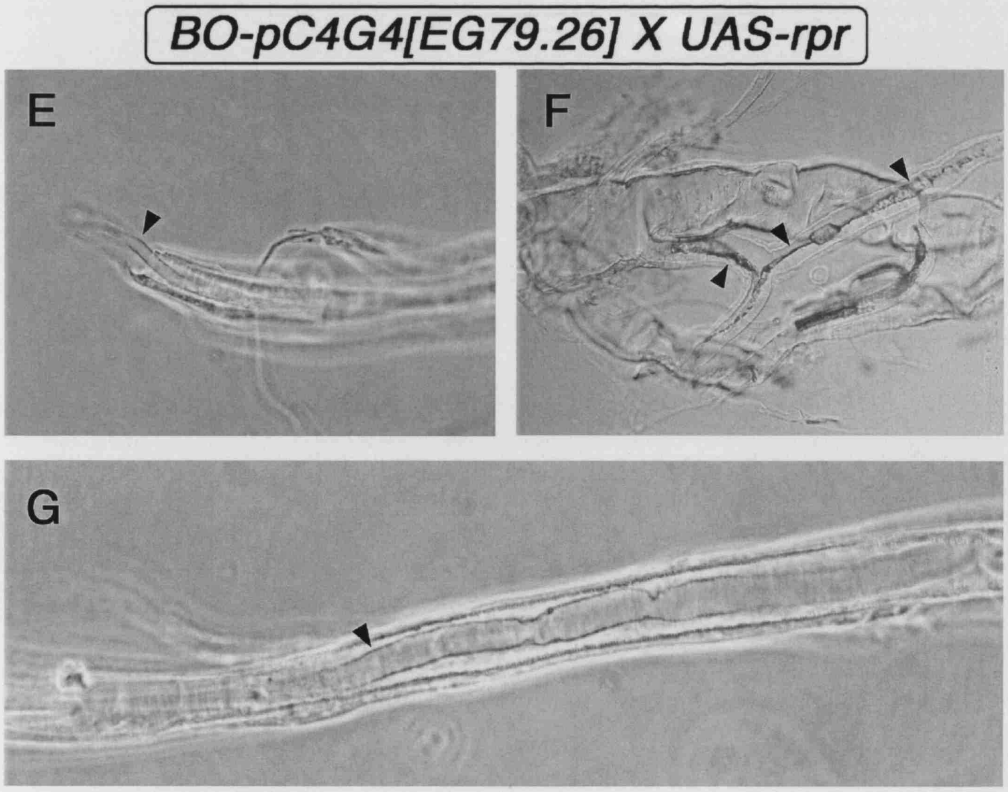

BO-pC4G4[EG79.26] X UAS-rpr
E
F
G

Figure 3.14 ***sna-GAL4[P0206]*** **and** ***Hnf4-GAL4[OK72]*** **Oenocyte-less Larvae Also Have Duplicated Mouth Hooks**

(A) L1 and L2 duplicated mouth hooks of a *sna>rpr* larva.

(B) L2 and L3 mouth hooks of a *OK72>rpr* larva.

sna-GAL4[P0206] x UAS-rpr

Hnf4-GAL4[OK72] x UAS-rpr

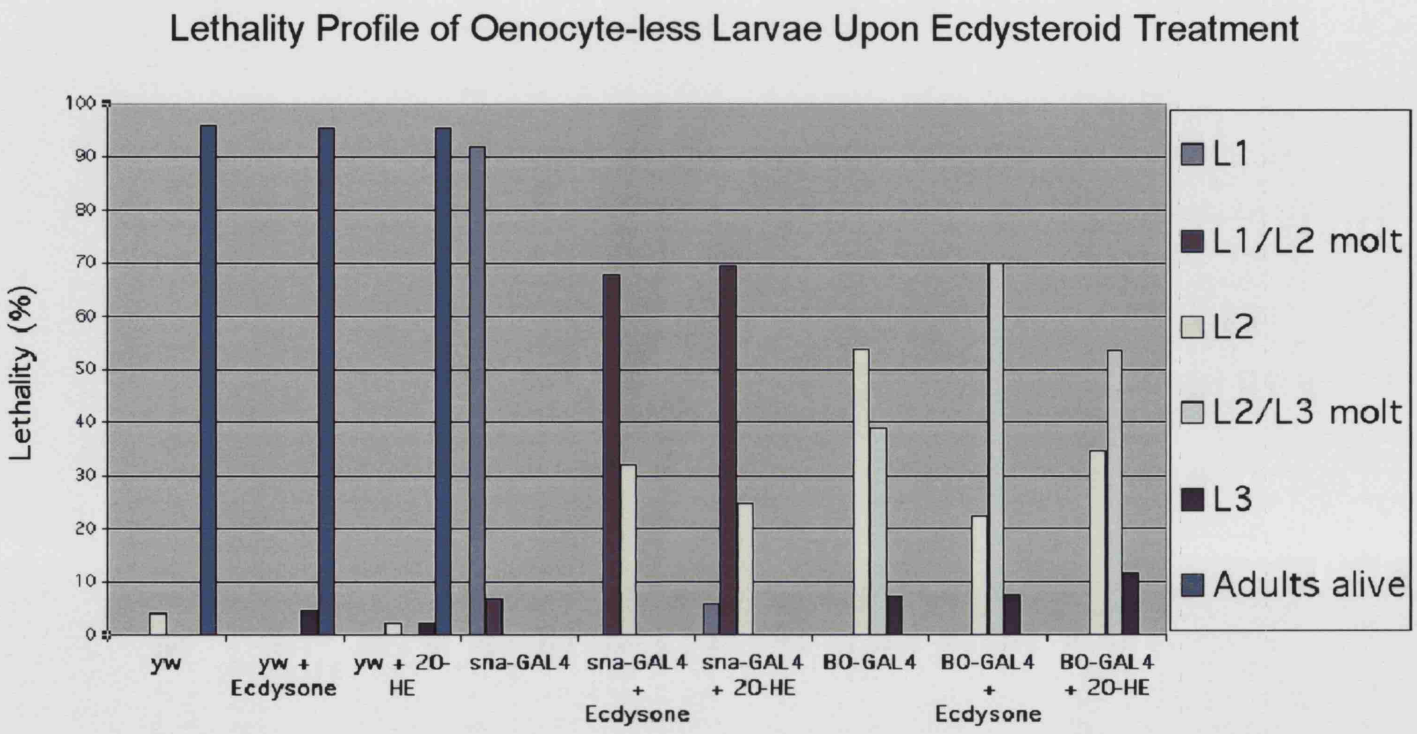
oenocytes could play a role, either in the conversion of inactive precursors to active ecdysteroids. A prediction of this hypothesis is that oenocyte-less larvae should be rescued by exogenous 20-E but not by ecdysone. Very little is known about ecdysteroid degradation, which might be essential for the steep-down phase of the pulses known to precede each moult (Riddiford 1993). One speculative possibility is that oenocytes are a major site for this degradation process. A prediction from this hypothesis is that provision of either exogenous ecdysone or 20-E would not rescue the observed phenotype and might even make it worse.

To begin to test these three ideas. larvae with or without oenocytes were fed on yeast paste containing ecdysone or 20-E. Controls included the use of ordinary yeast paste and control larvae that were: *yw x UAS-rpr* (as larvae in which oenocytes are present) and *sna-GAL4[P0206] x UAS-rpr* (as larvae in which neither ring gland nor oenocytes were present). Oenocyte-specific ablation was performed using *BO-GAL4[EG79.7] x UAS-rpr* larvae. The feeding regime used consisted of continuous provision of hormone throughout larval development. The resulting lethality profiles are compiled in Figure 3.15. Control larvae, having intact oenocytes, developed normally under any of the feeding conditions, producing fertile adults. Larvae in which both ring gland and oenocytes were eliminated (*UAS-rpr; sna-GAL4*) showed a highly reproducible arrest in L1, as expected for larvae lacking the ring gland. When larvae of this genotype were provided with either ecdysone or 20-E, a significant shift in lethality was observed with about one third of the larvae making it right through to L2 stages. Larvae in which oenocytes had been specifically removed (*UAS-rpr; BO-GAL4[EG79.7]*) have a polyphasic lethal profile, with half dying during L2 and a little less than that during the subsequent L2/L3 moult. A few specimens reach L3 but die before pupating. Upon administration of ecdysone or 20-E, a shift in the lethal profile is observed. With both treatments, the fraction of L2 entering the L2/L3 moult is somewhat increased but all animals die before exit into L3. The observed shift was slightly more pronounced for ecdysone than 20-E, however whether this is statistically significant or not remains to be confirmed.

The finding that ring gland-deficient larvae fed on ecdysone or 20-E were able to undergo the L1/L2 moult demonstrates that the hormone concentrations and feeding regime used were able to produce the expected physiological response. The observation that in both genotypes lacking oenocytes (*UAS-rpr; sna-GAL4* and *UAS-rpr; BO-GAL4[EG79.7]*) both ecdysone and 20-E produced similar responses argues

Figure 3.15 Lethality Profile of Oenocyte-less Larvae Treated with Ecdysteroids

Three different genotypes *(yw, sna-GAL4* and *BO-pC4G4[EG79.7])* were crossed to *UAS-rpr* and offspring was raised in the presence or absence of either ecdysone or 20-HE. Negative controls were *yw/UAS-rpr* larvae with and without ecdysteroids. *sna>rpr* larvae were used as positive controls for ecdysteroid action, as these larvae not only lack oenocytes but also ring gland. *BO>rpr* larvaes how a moderate shift in their lethal profile upon ecdysteroid treatment.


Lethality Profile of Oenocyte-less Larvae Upon Ecdysteroid Treatment
Lethality (%)
100
90
80
70
60
50
40
30
20
10
0
yw
yw + Ecdysone
yw + 20-HE
sna-GAL4
sna-GAL4 + Ecdysone
sna-GAL4 + 20-HE
BO-GAL4
BO-GAL4 + Ecdysone
BO-GAL4 + 20-HE
L1
L1/L2 molt
L2
L2/L3 molt
L3
Adults alive

against the hypothesis that larval oenocytes are a major active site for ecdysone to 20-E conversion. Moreover, as neither of the hormones tested could provide a complete rescue, the hypothesis that oenocytes solely act upstream of the ring gland is also unlikely. However, these experiments neither prove nor disprove the third hypothesis that oenocytes might be the site of 20-E degradation. Further experiments involving the use of lower doses of hormones and different feeding regimes might help to clarify these issues.

The ablation experiments described above strongly suggest that oenocytes play an essential role during larval life. Furthermore, they point towards an involvement in the moulting process. Nevertheless, due to the expression of *BO-GAL4* in the spiracular glands, the possibility exists that the phenotypes observed are caused by the ablation of this cell-type and the resultant retracted spiracles. However, using the additional driver *Hnf4-GAL4*, not expressed in the posterior spiracles, also produces arrested larvae with duplicated mouth hooks. This argues strongly in favour of oenocytes being associated with the *BO-GAL4* moulting phenotypes described here.

CHAPTER FOUR

Identification Of Genes Expressed In Oenocytes

CHAPTER FOUR: Identification Of Genes Expressed In Oenocytes

Identifying the repertoire of genes expressed by a particular cell type can often shed light upon its function. Prior to this work, very few genes were known to be expressed in oenocytes. Fewer still have been investigated in detail and most of these are involved in oenocyte induction, not function (reviewed (Elstob 2001; Elstob, Brodu et al. 2001; Brodu, Elstob et al. 2002). In this chapter I will describe the identification of 35 genes expressed in oenocytes using two complementary approaches. The first involves identifying genes by enhancer trapping, while the second takes advantage of a recently developed BDGP *in situ* database. Neither approach specifically identifies genes expressed in oenocytes and only in oenocytes but both do consistently pick out genes expressed in this cell-type and not in surrounding epidermal cells. Both approaches will be introduced and the genes identified will be described, grouping them according to their predicted biochemical functions. Finally, I will outline two recent strategies that are being used to ascribe *in vivo* functions to some of thesee genes, within the oenocyte context.

4.1 Fifteen Oenocyte Genes Identified From Enhancer Traps

Based on the analysis of reporter expression in oenocytes, a collection of P{*lacZ*}, P{*GAL4*} and P{*GFP*} enhancer traps was previously assembled in the lab (Elstob 2001). These were initially isolated in several large-scale enhancer trap screens carried out by BDGP, Flyview, Andrea Brand, Cahir O'Kane and Alex Gould. I was involved in rescreening many of these to eliminate non-oenocyte expressing lines and to determine whether oenocyte expression was in embryos, L3 larvae or both. The end result was a panel of 24 enhancer trap lines with *bona fide* expression in embryonic or larval oenocytes. The genomic point of insertion for 6 of the insertions from this collection had already been defined (*sal[A405] sal[P1340], svp[don1], Hnf4[P538], FALDH[P1342]* and *mirr[P880]*; reviewed in (Elstob 2001). To identify the insertion sites of the remaining 18 transposons, adjacent genomic sequences were recovered by inverse PCR and chain-termination sequencing (See Materials and Methods). 5'-P and 3'-P flanking sequences thus obtained were then BLASTN-searched against the *Drosophila* genome. Table 4.1 summarizes the loci targeted by each insertion, their associated phenotypes and the corresponding reporter expression patterns. Diagrams representing each of the insertions are shown in Section 4.3 (Figures 4.1 to 4.13).

Table 4.1 Gene Identity of 24 Enhancer Trap Lines

The line names are given according to the source of the line (Table 2.1). For insertion point data refer to Fig4.1-4.13 and Appendix 2.

E and L refers to embryonic and larval lethality respectively; ND refers to expression patterns not determined; SG is salivary glands; FB is fat body; ID is imaginal discs.

Information concerning expression patterns was collected in collaboration with Shilpa Mahadevaiah, Véronique Brodu, Phil Elstob and Alex Gould.

Gene	Line	Viability	Embryonic expression		Larval expression	
			Oenocytes	Other Tissues	Oenocytes	Other L3 Tissues
GAL4 traps						
EcR-A	P0197	lethal*	+	SG, posterior hindgut, apodemes (partly)	–	SG, FB, epidermis, wing disc, esophagus
Hnf-4	OK72	viable	–	SG, few cells in the gut	+	SG, epidermis, hindgut, fat body, some cells in the CNS, eye discs, proventricular zone
sna Tim17b2	P0206	viable	+ (st.13>)	SG, ring gland, epidermis	+	SG, ring gland, CNS, gut
CG4427	56B	viable	–	SG, some cells in the dorsal closure	+	Epidermis, other tissues
lacZ traps						
amn	P030	viable	–	Brain lobes, head region, epidermis, leg discs	+	Not Determined
amn	P0093	viable	–	Brain hemispheres	+	CNS, ring gland
amn	P0103	viable	+	Head region, epidermis	+	Only ring gland
amn	P0110	viable	–	–	+	Ring gland
CG11151	BI 12139	lethal	+	Amnioserosa, anterior spiracles, posterior spiracles, dorsal vessel, midgut	+	Not Determined
CG12424	P706	viable	++	Hindgut, oesophagus, dorsal vessel, ventral epidermis	–	Strong epidermis
dm	BI 12247	lethal	+ (st.13>)	Pharingheal muscles, antennomaxilary complex, gut, epidermis	–	–
Eip75B CG5290	P2103	lethal	–	–	+	SG, CNS, FB, ID, gonads, ring gland, epidermis, Malpighian tubules
FALDH	P1342 [1]	lethal	–	Maxillary lobes, hindgut, head, posterior region	+	SG (weak), histoblasts
FALDH	P2356	lethal	–	Epidermis, gut	+	–
Hnf4	P538 [1]	lethal	–	Almost nothing	+	Junction of Malpighian/gut (asymmetrical)
hnt	BI 11963	lethal	+ (st.13>)	Chordotonal organs, gut	+	Not Determined
mirr	P880 [1]	lethal (L)	++	CNS, dorsal epidermis	+	Wing and haltere discs, CNS, other?
psq	P0937	lethal	–	Anal plate, epidermis, amnioserosa, foregut	+	CNS, epidermis, ring gland, all ID, histoblasts
sal	A405 [1]	lethal	++ (st.10>)	CNS, epidermis, trachea	–	Wing disc, CNS, gut, trachea, posterior epidermis
sal	P1340 [1]	lethal	++	CNS, epidermis, trachea	+	Wing disc (pattern)
Transposon insertion	P1785		–	–	+	–
svp	S10 (don1)	lethal (E/L)	++ (st.10>)	CNS, dorsal vessel, 8th cluster	+	CNS, ring gland, fat body, discs, other?
svp, Trxl & ll	P890	lethal (L) **	++	Glia, CNS, head, heart, 8th cluster	+	CNS, a/p spiracle associated cells, other?
GFP trap						
Hnf4	L14A2	viable	–	–	+	Eye disc (sine oculis enhancer in construct), other?

*Lethality associated with another locus

** Multiple insertions: oenocyte pattern probably due to insertion at svp. Second insertion into the Trx complex

[1] Lines for which insertion point had been determined prior to this work

Detailed information concerning the sequences recovered for both ends of each P-element is given in Appendix 2.

Together, the enhancer trap studies identified 15 genes potentially expressed in oenocytes. The difference between the initial number of enhancer traps (24) and the final number of identified genes obtained (15) is due to the fact that several genes (*FALDH, Hnf4, sal* and *amn*; see below for abbreviations) were represented by multiple insertions (Table 4.1 and Section 4.3).

A lethal P insertion may result from transposition into enhancer or promoter elements of a gene, thus disabling transcription. Alternatively, insertion into intronic or coding sequences may result in mis-splicing and/or truncation of the protein itself. Of the total 24 insertions, 14 were associated with homozygous lethal chromosomes (Table 4.1), suggesting that these genes have an essential role. However, complementation tests are required to clarify this issue and rule out the possibility of lethality being caused by second-site mutations. Thus, approximately 50% of the oenocyte inserts are lethal, a percentage much higher than that find for random P-insertions (~10%). The reasons for this discrepancy are not totally clear but it may be that enhancer traps entered into databases are already biased in favour of lethal insertions. Interestingly, the only viable traps found in coding regions represent 4 independent insertions into the *amnesiac (amn)* locus (Figure 4.13). These results are consistent with the observation that *amn* null alleles are homozygous viable (Moore, DeZazzo et al. 1998).

4.2 Twenty-one Oenocyte Genes Found by Searching BDGP *In Situ* Screen

Several large-scale screens, taking advantage of the completed genome sequence, have been recently established in *Drosophila*, including saturation by different transposable elements, study of dsRNA in cell culture assays or *in silico* comparisons of complete gene families across species. One ongoing approach consists of the semi-automated *in situ* hybridization of embryos with sequenced, full-length, non-redundant cDNAs representing near 11,000 *Drosophila* genes (Drosophila Gene Collections 1.0 and 2.0, Tomancak, Beaton et al. 2002). A public database, containing embryonic images of all the *in situ* patterns became accessible via the BDGP website during the course of my Ph.D. studies. This useful resource was therefore used to search for all genes with an oenocyte expression pattern in embryos. This approach yielded 21 different oenocyte genes and one transposable element (GH06606), see

Appendix 3). Six of these genes (*Atet, Cat, Cpr, Cyp4g1, nesprin* and *NH3*; see below for full names) have previously been genetically characterised to at least some degree, while the remaining 15 represent novel predicted genes. Interestingly, expression of 4 of these genes (*CG14615, CG18609, Cyp4g1* and *CG17562*) appears to be completely restricted to oenocytes during embryogenesis while three more also seems to be highly specific for this cell type (*CG6921, CG11567, CG31095*).

4.3 A Total of 35 Oenocyte Genes Identified in Both Screens

The combined results of both types of screens undertaken led to the classification of 35 oenocyte genes into different categories based on the types of proteins they encode them (Table 4.2). All novel genes were compared by BLASTP searches with the protein database. The results of this categorisation of predicted functions includes:

- 2 Lipophorin receptors involved in the capture of large lipoprotein complexes
- 5 FA-microsomal enzymes, 4 of which are known to be involved in initial modification steps required for subsequent degradation by peroxisomes
- 6 Peroxisomal proteins (5 lipid-metabolic enzymes and one ABC transporter)
- 3 Additional lipid-metabolising enzymes not falling into any of the previous categories
- 4 Members of the nuclear-hormone receptor gene family

In addition, 8 other transcription factors were identified, many of which are strong candidates to be involved in the early steps of oenocyte induction and differentiation. Finally, 7 remaining genes could not be clearly assigned to any of the previous classes and their precise function in oenocytes is yet to be established. Importantly, from a total of 35 genes, 15 represent predicted genes that have not been classified at the functional genetic level.

A brief gene-by-gene survey of the loci found now follows, highlighting the site of transposon insertion, the nature of the products encoded and, if determined, their expression patterns. For previously characterised genes, stress will be placed on what is known in the context of *Drosophila*. For the novel *Drosophila* genes, comparisons will be made to close orthologs in other species.

Table 4.2 Oenocyte Genes Recovered from Both Screens

Genes found in the enhancer trap screen are coloured blue, those found in the *in situ* screen are green, while the only gene found in both approaches is marked in red.

35 Oenocyte Genes Identified in Both Screens

- VLDL/LDL membrane receptors
  - *Lipophorin receptor 1 (LpR1) CG4861-CG31095*
  - *Lipophorin receptor 2 (LpR2) CG31092*

- Microsomal Enzymes
  - *Cytochrome 4g1 (Cyp4g1)*
  - *Cytochrome P450 reductase (Cpr)*
  - *CG18609 FA Elongase*
  - *CG6921 FA Elongase*
  - *Fatty aldehyde dehydrogenase (FALDH)*

- Peroxisomal Proteins
  - *ABC transporter expressed in trachea (Atet)*
  - *CG9527 Pristanoyl-CoA oxidase*
  - *Catalase (Cat)*
  - *CG11151 Sterol Carrier Protein 2 (SCP2-like)*
  - *CG17562 3ß-hydroxysteroid dehydrogenase (3ß-HSD)*
  - *CG12428 Carnitine-O-octanoyltransferase (COT)*

- Other lipid enzymes
  - *CG12262 Acyl-CoA-dehydrogenase*
  - *CG14615 Acyl-CoA-N-acyltransferase*
  - *CG7920 4-hydroxybutyrate CoA-transferase*

- Nuclear hormone receptors
  - *Ecdysone-induced protein 75B (Eip75B)*
  - *Ecdysone receptor isoform A (EcR-A)*
  - *Hepatocyte nuclear factor 4 (Hnf-4)*
  - *seven-up (svp)*

- Other Transcription factors
  - *pipsqueak (psq)*
  - *snail (sna)*
  - *diminutive (dm)*
  - *mirror (mirr)*
  - *hindsight (hnt)*
  - *spalt (sal)*
  - *CG12424 pnt-like TF*
  - *CG4427 C2H2-type Zn-finger TF*

- Miscellaneous proteins
  - *CG3132 ß-galactosidase* [Lysosomal protein]
  - *amnesiac (amn)* [Secreted molecule]
  - *Na+ H+ exchanger 2 (NHE2)* [Membrane bound transporters]
  - *MSP-300/nesprin* [Actin binding protein]
  - *CG31361* [Immunoglobulin domain-containing protein]
  - *CG3328* [Unknown function, no known protein motif]
  - *CG31764* [Unknown function, no known protein motif]

4.3.1 *CG4861-CG31095* and *CG31092*, Two Adjacent Lipophorin Receptors

Two adjacent genes *(CG31092/LpR2* and *CG31095)* at 96Fwere both expressed in oenocytes and CNS (Appendix 3). They are both highly related to Lipophorin receptors genes. *LpR2* orthologs in other insects have been postulated to encode receptors for lipophorin that mediate its endocytosis, hence their name (Dantuma, Potters et al. 1999; Cheon, Seo et al. 2001). Lipophorin acts as a yolk protein precursor and constitutes an abundant lipoprotein component of hemolymph. Phylogenetic analysis has revealed that *LpRs* are members of the low-density lipoprotein receptor (LDLR) family and it has been suggested that the insect LpRs and vertebrate LDL/VLDL receptor lineages derive from a vitelogenin receptor VgR-like ancestor. Recently, *CG31094* and *CG31092/LpR2* were cited to encode LpRs (Culi and Mann 2003). Surveying release 3 of the fly genome, I was only able to find *CG31095* adjacent to LpR2; no *CG31094* was present in this release of the genome. However, *CG31095* and adjacent *CG4861* are predicted to produce a single transcript with high similarity to the adjacent gene *LpR2*. Therefore, it is probable that *CG4861-CG31095* is what elsewhere was refered to as *LpR1* or *CG31094*. Importantly, both *LpR1* and *LpR2* encode a receptor with the same general structure as vertebrate LDL/VLDL receptors. They contain six and eight cysteine-rich binding repeats at the N-terminus, respectively, followed by an EGF-like domain and YWTD domains.

In vertebrates, LDLR is the prototype lipoprotein receptor expressed in tissues that utilize lipoproteins (See Introduction, Section 1.2.1). VLDLR is distinctly expressed in tissues that metabolize VLDL-derived free fatty acids, indicating a function of the receptor in delivering triglyceride-rich lipoproteins to target tissues. Particles internalized via LDLR are delivered to lysosomes for degradation, where the apolipoproteins are broken down into amino-acids while the lipids are released into the cytosol (reviewed in (Willnow 1999). The finding that *LpR1* and *LpR2* are expressed in oenocytes raises the possibility that expression of LDLR-like molecules in oenocytes may mediate the uptake of various lipoprotein conjugates from the surrounding hemolymph.

4.3.2 Microsomal Enzymes

Cytochrome P450 4G1 (Cyp4g1, CG3972)

CG3972 has been named *Cyp4g1*, based on the closest homology of its predicted product to vertebrate cytochrome P450s of the Cyp4 family (See Introduction, Section 1.5.5). *In situ* hybridisation during embryonic stages showed that remarkably, this gene appears to be exclusively expressed in oenocytes (Appendix 3). Immunolocalization further confirmed that this gene is exclusively expressed in oenocytes during embryonic stages (Figure 4.1.A-D) and extended this restricted expression to L1 stage (Figure 4.1.E-H). Subcellular localization appeared to be limited to some cytoplasmic subcellular organelles, compatible with the ER localization of other members of this family of enzymes (Section 1.5.5). In vertebrates, the *Cyp4* family encodes P450 enzymes capable of hydroxylating the terminal ω-carbon and to a lesser extent, the ω-1 position of both saturated and unsaturated fatty acids, as well as enzymes active in the ω-hydroxylation of various prostaglandins. Cyp4 genes, are induced in rat liver and kidney by PPs and this may well be mediated via PPARs (Section 1.3.2 and Section 1.4). Thus one attractive hypothesis is that Cyp4g1 may be upregulated by a PPAR-like receptor in oenocytes. In addition, microarray experiments have recently revealed that *Cyp4g1* appears to be upregulated in hypoxic larvae (Zhou, Lambert et al. 2003), perhaps consistent with one of the classic hypotheses that state that oenocytes play a role in O_2 regulation (Section 1.7.4).

Cytochrome P450 reductase (Cpr or CG11567)

One of the seven most oenocyte-specific genes was *Cytochrome P450 reductase (Cpr or CG11567). Cpr* encodes a predicted NADPH–cytochrome P450 oxidoreductase involved in electron transport (Hovemann, Sehlmeyer et al. 1997). Based on its sequence, *Cpr* has been proposed to be a component of the ER (Flybase FBgn0015623). *Cpr* is expressed at high levels in adult antennae and may be required in the process of olfaction, participating together with P450s in the degradation of odor molecules once they have activated their receptors (Hovemann, Sehlmeyer et al. 1997). Its function during embryonic and larval life remains to be established but there is clearly the potential for a biochemical link between Cpr and Cyp4g1.

Figure 4.1 Cyp4g1 is Exclusively Expressed in Oenocytes During Embryonic and Early Larval Life

Wild-type embryos and larvae of these panels were immunolabelled against Cyp4g1 (red).

(A-D) Stage 17 wild-type embryo. Background staining is shown in green in Panel (A) to outline the shape of the embryo with respect to Cyp4g1 expression. (C-D) Two adjacent single sections of two oenocyte clusters showing cytoplasmic Cyp4g1 localization.

(E-H) Early L1 larva showing oenocyte-restricted Cyp4g1 expression. Panels (F-H) show high power single sections of three oenocyte clusters. Cyp4g1 localize to a cytoplasmic compartment.

Stage 17 embryo
Early L1 larva
A
B
C
D
E
F
G
H

CG6921* and *CG18609 (Two Long Chain-Fatty Acid Elongases, ELOs)

Two other members of the seven most oenocyte-specific genes are *CG6921* and *CG18609,* both encoding members of the family of ER-located enzymes involved in long-chain FA elongation. Mutations in a member of this family (human *ELOVL4*) have been associated with human macular dystrophy (Zhang, Kniazeva et al. 2001) and, more recently, *C. elegans ELO-2* has been identified as the enzyme mediating the first step of palmitic acid (C16:0) elongation (Kniazeva, Sieber et al. 2003). Interestingly, RNAi-mediated downregulation of *ELO-2* causes an imbalance in FA composition, which leads to developmental defects including slow growth and small body size. These phenotypes are strikingly reminiscent of some of the phenotypes displayed by oenocyte-less *Drosophila* larvae (See Section 3.4). Genetic and biochemical characterisations of *Drosophila CG6921* and *CG18609 ELOs* have yet to be performed.

Fatty aldehyde dehydrogenase (FALDH)

Two lethal *lacZ* enhancer traps, l(2)03610 and l(2)08717, were found to be inserted in the same intron, common for all isoforms of *Fatty aldehyde dehydrogenase (FALDH)*, also called *Aldehyde dehydrogenase type III (AldhIII)* (Figure 4.2). *FALDH* codes for a microsomal enzyme predicted to be involved in the conversion of hexadecanal to palmitate (van Veldhoven and Mannaerts 1993; Kelson, Secor McVoy et al. 1997; Rizzo 1998). *In situ* hybridizations for *FALDH* reveal widespread expression in late embryonic stages, including endoderm and mesoderm, with high levels in the hindgut and maxillary bud (data not shown). This embryonic expression pattern was similar to the one observed with the enhancer trap lines, confirming the identity of the gene trapped and suggesting that FALDH is not expressed in oenocytes until larval stages. Interestingly, FALDH is required for the synthesis of PE, the major phospholipid of insect membranes, whose levels act as a sensor regulating FA and phospholipid synthesis (Section 1.2.3).

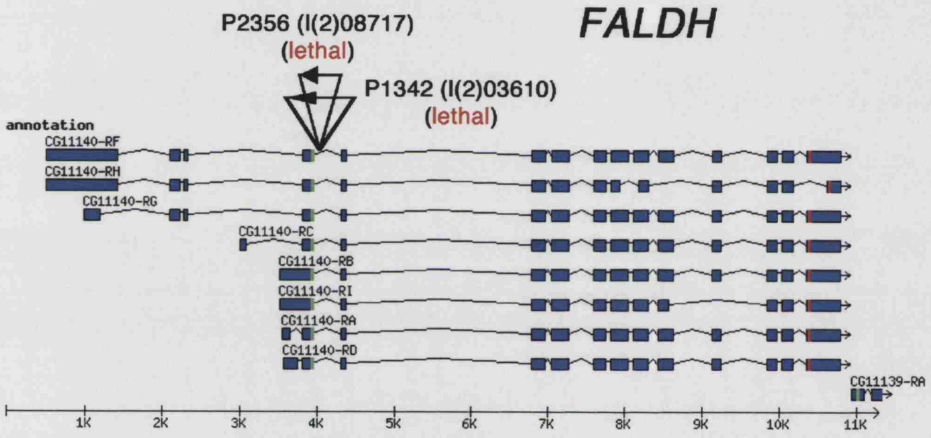
4.3.3 Peroxisomal Proteins

Predicted products for 6 oenocyte genes were found to share similarities with conserved peroxisomal proteins in other species. Although, the exact mechanisms by which resident proteins are shuttled to peroxisomes are not fully understood, either of two peroxisomal-targeting sequences, called PTS1 and PTS2, is often involved (Section 1.3.1). Thus, I compared the sequences of these 6 putative peroxisomal

Figure 4.2 The *Fatty Aldehyde Dehydrogenase (FALDH)* Locus

In this and all subsequent figures showing the insertion of enhancer elements , both the line number and the name of the allele (in brackets) are given. For precise insertion point data for this and other figures refer to Appendix 2.

l(2)03610 and l(2)08717 enhancer traps are both inserted into an intronic region common for all *FALDH* isoforms.


FALDH
P2356 (l(2)08717)
(lethal)
P1342 (l(2)03610)
(lethal)
annotation
CG11140-RF
CG11140-RH
CG11140-RG
CG11140-RC
CG11140-RB
CG11140-RI
CG11140-RA
CG11140-RD
CG11139-RA
1K
2K
3K
4K
5K
6K
7K
8K
9K
10K
11K

proteins to consensus sequences for PTS1 and PTS2. Remarkably, all six of them contain sequences either in complete agreement with, or with one-mismatch to these two consensus sequences (Table 4.3). In three cases (*CG9527, Cat, CG11151*), both PTS1 and PTS2 are present within the sae protein. The fact that the 6 putative peroxisomal proteins contain PTSs strengthens the possibility that really are resident peroxisomal components. However, to determine unequivocally their exact subcellular localization, direct evidence by immunocytochemistry has yet to be provided.

ABC transporter expressed in trachae (Atet)

The *ABC transporter expressed in trachae (Atet)* gene has been cloned and is reportedly expressed in embryonic trachae and amnioserosa (Kuwana, Shimizu-Nishikawa et al. 1996). This expression pattern differs from that deposited in the BDGP embryonic *in situ* database, which includes oenocyte expression (Appendix 3). The ATP-binding cassette (ABC) transporter superfamily contains proteins that translocate a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids, sterols and drugs. The *Atet* product belongs to the ABCG subfamily, with each gene encoding half-transporters that can act as homodimers or heterodimers (reviewed in (Dean, Hamon et al. 2001; Dean, Rzhetsky et al. 2001). This family also includes the *Drosophila white* gene product, involved in the transport of eye pigments. The closest ortholog to *Atet* in the human genome is *ABCG1*. Interestingly, mammalian ABCG1 protein, is involved in cholesterol transport (reviewed in (Dean, Hamon et al. 2001; Dean, Rzhetsky et al. 2001). The function and biochemical specificity of *Drosophila Atet* remain to be characterised.

Pristanoyl-CoA oxidase (CG9527)

CG9527 encodes a pristanoyl-CoA oxidase, a type of branched-chain acyl-CoA oxidase. Vertebrate orthologs of this gene are also known as peroxisomal acyl-Coenzyme A oxidase 3 (Acox3). This type of peroxisomal acyl-CoA oxidase functions in part of the cycle of ß-oxidations leading to the degradation of branched-chain FAs and straight LCFA and VLCFA (Section 1.3.2 and Figure 1.2). 2-Methyl-branched acyl-CoAs are degraded in hepatocyte peroxisomes via pristanoyl-CoA oxidase, multifunctional protein-2 (MFP-2) and sterol carrier protein-X (SCPX). *CG9527* is highly expressed in the embryonic midgut and the oenocytes, suggesting

Table 4.3 Peroxisomal Targeting Sequences (PTS1 and PTS2) of Proteins Expressed in Oenocytes

x refers to any amino acid; > refers to the carboxy-terminus of the protein; < refers to the amino-terminus of the protein; {number} refers to the number of aminoacids between sequences at each side of the brackets; [XX] refers to all aminoacids compatible with the consensus for a particular position.

Gene \ Motif	PTS1	PTS2
	xKL>; xKF>; SxL>; SxF>; SKx>	<Mx{0-60}[RK][LVI]xxxxx[QH][LA]
ABC transporter expressed in trachea		<Mx {8} lVxxxxxHA <Mx{343}RLxxxxxHL
CG9527 Pristanoyl-CoA oxidase	AKL	<Mx{55} RLxxxxxQw <Mx{59} KVxxxxxQh
Catalase	SKF	<Mx{63} RIxxxxxHA
CG11151 Sterol Carrier Protein 2	AKL	<Mx{30} KIxxxxxvA <Mx{59} KVxxxxxvA <Mx{75} aLxxxxxQA
CG17562 3ß-hydroxysteroid dehydrogenase		<Mx{52} RIxxxxxdA <Mx{127}RaxxxxxQL <Mx{262}RIxxxxxHA
CG12428 Carnitine-O-octanoyltransferase	SKL	

that these two tissues may be highly active in processing long and branched-chain FAs.

Catalase (Cat)

The *Catalase (Cat)* gene is expressed at high levels in the oenocytes at late embryonic stages (Appendix 3). This hemoprotein catalyses the conversion of hydrogen peroxide to water and oxygen and is a component of all known peroxisomes. In fact, it is used in many species as a diagnostic marker for these organelles (Section 1.3.2). In *Drosophila,* amorphic mutations in the *Cat* gene are hypersensitive to hydrogen peroxide with adult homozygotes dying just after eclosion (Griswold, Matthews et al. 1993). Although *Cat* overexpression does not prolong life span, nor provide improved protection against oxidative stress induced by hyperoxia or paraquat treatment, it can enhance resistance to exogenous hydrogen peroxide (Orr and Sohal 1992). Two developmental peaks of enzymatic activity have been detected, the smaller in late L3, just prior to puparium formation, and the larger during metamorphosis (Orr, Orr et al. 1996). Consistent with this, *Cat* expression is known to be responsive to ecdysone and exhibits both transcriptional and post-translational regulation (Radyuk, Klichko et al. 2000).

Sterol Carrier Protein 2-like (CG11151)

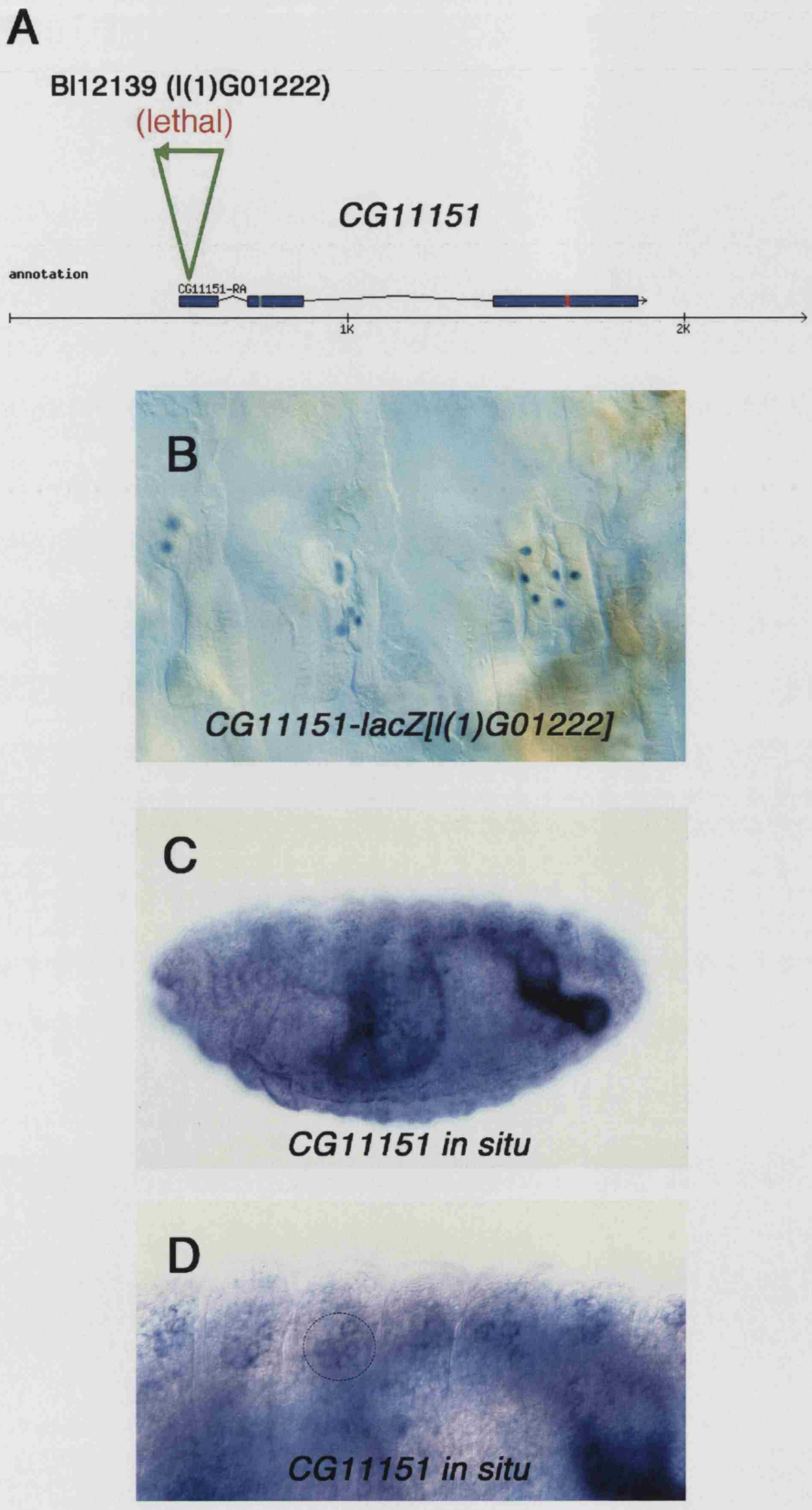
A lethal insertion (l(1)G0122) was found in the first, non-coding, exon of the predicted gene *CG11151* (Figure 4.3A). This P{*lacZ*} trap is expressed in oenocytes both during embryonic stages and L3 (Table 4.1 and Figure 4.3B). CG11151 was the only oenocyte gene found in the enhancer trap screen and also in the BDGP *in situ* screen. In *situ* hybridization of *CG11151* by myself and BDGP showed that the enhancer trap recapitulates the expression of the endogenous gene, including expression in the midgut and oenocytes (Table 4.1, Figure 4.3C-D and Appendix 3). Expression in embryonic oenocytes begins late, only becoming visible at stage 17. BLASTP-searches revealed that this gene contains a sterol carrier protein 2 (SCP2) domain. The most similar database matches to the *CG11151* predicted protein are the rat 17ß-hydroxysteroid dehydrogenase type IV (17ß-HSD IV) and the rat peroxisomal multifunctional ß-oxidation protein 2 (MFP-2). These two names represent the same enzyme, also known as D-bifunctional protein (D-BP), 3-hydroxyacyl-CoA dehydrogenase or SCP2 (Introduction Section 1.3.2 and Figure 1.2). 17ß-HSD IV

Figure 4.3 Genomic Map and Expression Pattern of *CG11151-Sterol Carrier Protein 2 (SCP2)*

(A) Genomic map of *CG11151-Sterol Carrier Protein 2 (SCP2)* indicating the insertion point of the l(1)G0122 enhancer trap into the first non-coding exon of *SCP2.*

(B) Three oenocyte clusters from a dissected pelt of an *SCP2-lacZ [l(1)G0122]* L3 larva stained with X-gal. Oenocyte nuclei are labelled.

(C-D) I*n situ* hybridization for *CG11151* in a stage 17 wild-type embryo. Expression is observed in gut (C) and oenocytes (C and dotted circle in D).


A
Bl12139 (l(1)G01222)
(lethal)
CG11151
annotation
CG11151-RA
1K
2K
B
CG11151-lacZ[l(1)G01222]
C
CG11151 in situ
D
CG11151 in situ

enzyme has been described as containing a specific unidirectional oxidative 17ß-HSD activity, catalysing the conversion of steroid molecules, such as ß-estradiol to oestrone (Adamski, Normand et al. 1995). D-BP has been associated with the liver degradation of straight-chain and 2-methyl-branched chain fatty acyl-CoA esters via peroxisomal ß-oxidation (Section 1.3.2). D-BP functions either as a homodimer, or can be proteolytically cleaved to two functional polypeptides of 46-kDa and 36-kDa, the latter containing the SCP2 domain. Another vertebrate protein with similarities to *CG11151* is SCPx, an enzyme acting in the next step after D-BD action on peroxisomal FA ß-oxidation (Introduction Section 1.3.2 and Figure 1.2). SCPx is composed of an N-terminal 3-oxoacyl-CoA thiolase domain and a C-terminal SCP2 domain (reviewed in (Hashimoto; Mannaerts, Van Veldhoven et al. 2000). Analysis of human *SCPx* and *SCP2* revealed that both proteins are encoded by alternative splicing from the same locus (reviewed in (Wanders 2000). Similar to D-BD, the SCPx polypeptide is split *in vivo* into thiolase and SCP2 (Antonenkov, Van Veldhoven et al. 1997); reviewed in (Mannaerts, Van Veldhoven et al. 2000).

Although the name of SCP2 is reminiscent of sterol carrier protein, SCP2 does not bind to lipids specifically or stoichiometrically. Instead, SCP2 facilitates the formation of peroxisomal multienzymatic complexes containing the rate-limiting enzyme acyl-CoA oxidase (Figure 1.2, (Bun-ya, Muro et al. 2000). Hence, SCP2 can be considered as a peroxisomal molecular chaperone involved in FA ß-oxidation.

3ß-hydroxysteroid dehydrogenase-like (CG17562)

Another one of the 7 most oenocyte-specific genes was *CG17562.* According to BDGP it is exclusively expressed in oenocytes, at least during embryogenesis. BLASTP-searches revealed similarities to proteins containing a 3ß-hydroxysteroid dehydrogenase-like (3ß-HSD-like) domain. In vertebrates, 3ß-hydroxysteroid dehydrogenase/isomerase (3ß-HSD) is an enzyme essential for the biosynthesis of all active steroid hormones. 3ß-HSD consists of multiplevariants, each encoded by a different gene and with different spatiotemporal expression (reviewed by (Peng, Arensburg et al. 2002). 3ß-HSD enzymes have been found in different subcellular compartments, depending on the tissues and species involved (Pozzi, Lantos et al. 1997; Pelletier, Li et al. 2001). I found that *CG17562* contains 3 copies of a PTS2 motif, suggesting that in *Drosophila* it may be peroxisomal. At present, the substrate specificity of the *CG17562* product is not known. However, it is interesting that

isoform A of EcR, the *Drosophila* 20-E receptor (Introduction Section 1.6.4), is also expressed in oenocytes, raising the possibility that precursors, active ecdysteroids or their metabolites are modified by *CG17562* activity.

Carnitine-O-octanoyltransferase COT (CG12428)

CG12428 encodes a Carnitine-O-octanoyltransferase (COT). COT, a peroxisomal component, transesterifies medium- and long-chain acyl-CoA molecules into acylcarnitines for further transport to mitochondia (Introduction 1.3.2 and Figure 1.2). *CG12428* contains a PTS1 motif, consistent with its homology to known peroxisomal COTs in vertebrates. In *Drosophila*, there are another three COTs (*CG5122, CG5265, CG1041*), none of which have yet been characterised at the level of expression pattern.

4.3.4 Other Lipid Enzymes

Acyl-CoA-dehydrogenase (CG12262)

BLASTP-search against the predicted gene *CG12262* revealed similarity to the mitochondrial *medium chain Acyl-CoA dehydrogenase (MCAD)* and also to mitochondrial *short chain acyl-CoA dehydrogenase (SCAD, Butyryl-CoA dehydrogenase)*. The enzymes encoded by these two genes account for two of the four acyl-CoA dehydrogenases that participate in FA ß-oxidation (Introduction Section 1.3.2). A murine short chain acyl-CoA dehydrogenase (Acads) has been studied in the context of the hippocampus and the liver. This enzyme, catalyses the first step in the ß-oxidation of C4-C6 fatty acids and mutations in the corresponding gene increase ketone-body production in the liver via the overproduction of acetyl-CoA, formed by ß-oxidation of long- and medium-chain fatty acids (Tafti, Petit et al. 2003).

Acyl-CoA N-acyltransferase (CG14615)

CG14615 is another of the 7 most oenocyte-specific genes, which according to BDGP is exclusively expressed in oenocytes during embryogenesis. The only known distant vertebrate relative to the predicted protein of *CG14615* is human BXMAS2-10 (E value of 0.18), a protein isolated from acute myelogenous leukaemia

bone marrow (Accession Number AAH21682). A *Drosophila* distant relative with no evident sequence similarity, aaNAT, is also selectively expressed in oenocytes during embryogenesis (Hintermann, Grieder et al. 1996). aaNAT is capable of acetylating dopamine, tryptamine and the immediate melatonin precursor serotonin in COS cells. Domains of the *CG14615* predicted protein include GCN5-related N-acetyltransferase and Acyl-CoA N-acyltransferases (NAT). The GCN5-related N-acetyltransferase family is listed in InterPro as compriseing proteins involved in phosphorylation, adenylation or acetylation of aminoglycosides. The NAT family comprises enzymes involved in the conjugation of FAs and steroids to amino groups of amino-acids and peptides. Subcellular localization studies of members of the NAT family have categorised them as being either cytosolic, microsomal or peroxisomal. Because of the low similarity of the *CG14615* product to proteins containing these domains, the subcellular localisation and reactions catalysed by this enzyme remain to be established.

4-hydroxybutyrate CoA-transferase (CG7920)

The predicted product of *CG7920* shares no significant similarity to any vertebrate protein in the databases. However, I did find relatives in bacteria and in the *C. elegans* genome. In all these species, *CG7920*-like genes encode 4-hydroxybutyrate CoA-transferases. For example, in bacteria *Clostridium aminobutyricum*, *4-hydroxybutyrate CoA-transferase (abfT)* promotes 4-hydroxybutyrate degradation, by catalysing the formation of a linkage between 4-hydroxybutyrate and Coenzyme A (Gerhardt, Cinkaya et al. 2000). This allows fermentation of 4-aminobutyrate via succinic semialdehyde, 4-hydroxybutyrate, 4-hydroxybutyryl-CoA and crotonyl-CoA to acetate and butyrate. Due to the high degree of similarity between the *CG7920* and *abfT* products (65%), it is likely that they play similar roles. This potentially links *CG7920* with Co-A reactions, an emerging theme common to many of the oenocyte lipid-metabolic genes. The subcellular localization of *CG7920* remains to be established.

4.3.5 Nuclear Hormone Receptors

Ecdysone-induced protein 75B (Eip75B)* and *CG5290

A P{*lacZ*} lethal enhancer trap (l(3)rL061) was inserted between the predicted gene *CG5290* and also *Ecdysone-induced protein 75B (Eip75B*, Figure 4.4). *CG5290* contains a small tetratricopeptide repeat domain (TPR) involved in a variety of functions, including protein-protein interactions. *CG5290* expression was analysed by *in situ* hybridization in embryos, revealing a ubiquitous pattern that contrasts with no expression of the P{*lacZ*} line at these stages (Table 4.1 and data not shown). Recently, other P-reporter lines inserted closer than l(3)rL061 and within *Eip75B* have been shown to be expressed in oenocytes (Hayashi, Ito et al. 2002), suggesting that l(3)rL061 reflects the expression of *Eip75B* rather than *CG5290.* Three nuclear hormone receptors (E75A, E75B and E75C) are encoded from the E75 ecdysteroid-inducible gene of the 75B early puff (Segraves and Hogness 1990). In *Drosophila*, ecdysteroid-induced *Eip75B* participation in a positive feedback loop amplifying or maintaining ecdysteroid titre during larval development and thus ensuring proper progression from moult-to-moult (Bialecki, Shilton et al. 2002). The embryonic and larval expression of *Eip75B* has not yet been reported. However, isoform A-specific mutations have a reduced ecdysteroid titer during larval development, resulting in developmental delays, arrests and moulting defects. Interestingly, these phenotypes are strikingly similar to those seen in oenocyteless larvae (Chapter 3).

Traditionally, comparisons of *Eip75B*, biased towards the DNA Binding Domain (DBD), have categorized it as the *Drosophila* ortholog of vertebrate *Rev-Erb* genes (Thummel 1995; Maglich, Sluder et al. 2001). *Rev-Erb alpha* is expressed in adipose tissue, skeletal muscle and brain (Lazar, Hodin et al. 1989) and it has been shown to be a direct target of PPARγ (Fontaine, Dubois et al. 2003). Using BLASTP, I re-examined the similarity of Eip75B isoforms to the predicted mouse proteome. I found that, although the DBD of Eip75Bs are indeed most similar to that of Rev-Erb, when taken as full-length proteins, all Eip75B isoforms are most similar to vertebrate PPARs (Table 4.4). This finding may have particular relevance to oenocytes, as PPARs are central regulators of lipid metabolism in vertebrates (Introduction Section 1.4).

Ecdysone receptor isoform A (EcR-A)

A lethal P{*GAL4*}, P0197, was inserted into the non-coding first exon of the isoform A transcript of the *Ecdysone receptor (EcR)* gene (Figure 4.5A). *EcR* encodes

Figure 4.4 The *Ecdysone Induced Protein 75B (Eip75B)* and *CG5290* Loci

The insertion point of l(3)rL061 enhancer trap between these two loci is indicated.

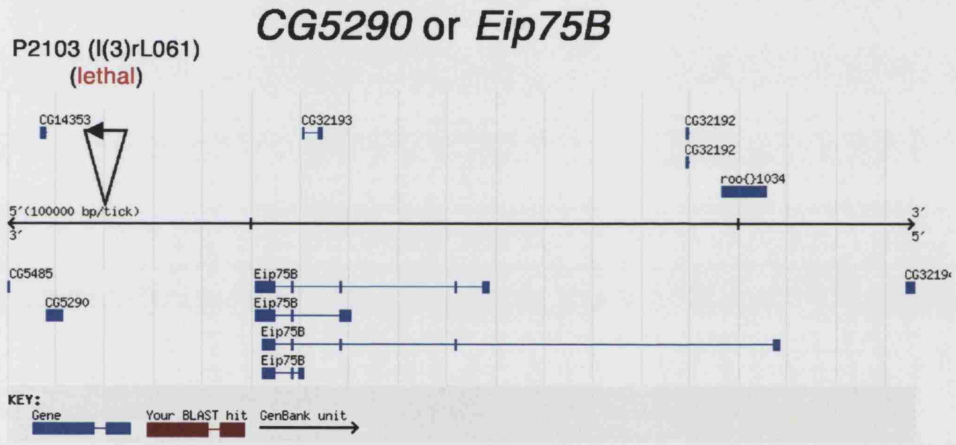

CG5290 or Eip75B
P2103 (l(3)rL061)
(lethal)
CG14353
CG32193
CG32192
CG32192
roo()1034
5′(100000 bp/tick)
3′
3′
5′
CG5485
CG5290
Eip75B
Eip75B
Eip75B
Eip75B
CG3219
KEY:
Gene
Your BLAST hit
GenBank unit

Table 4.4 E Values of BLAST-P Searches With Full-Length *Drosophila Eip75B* Products Compared Against the Mouse Genome

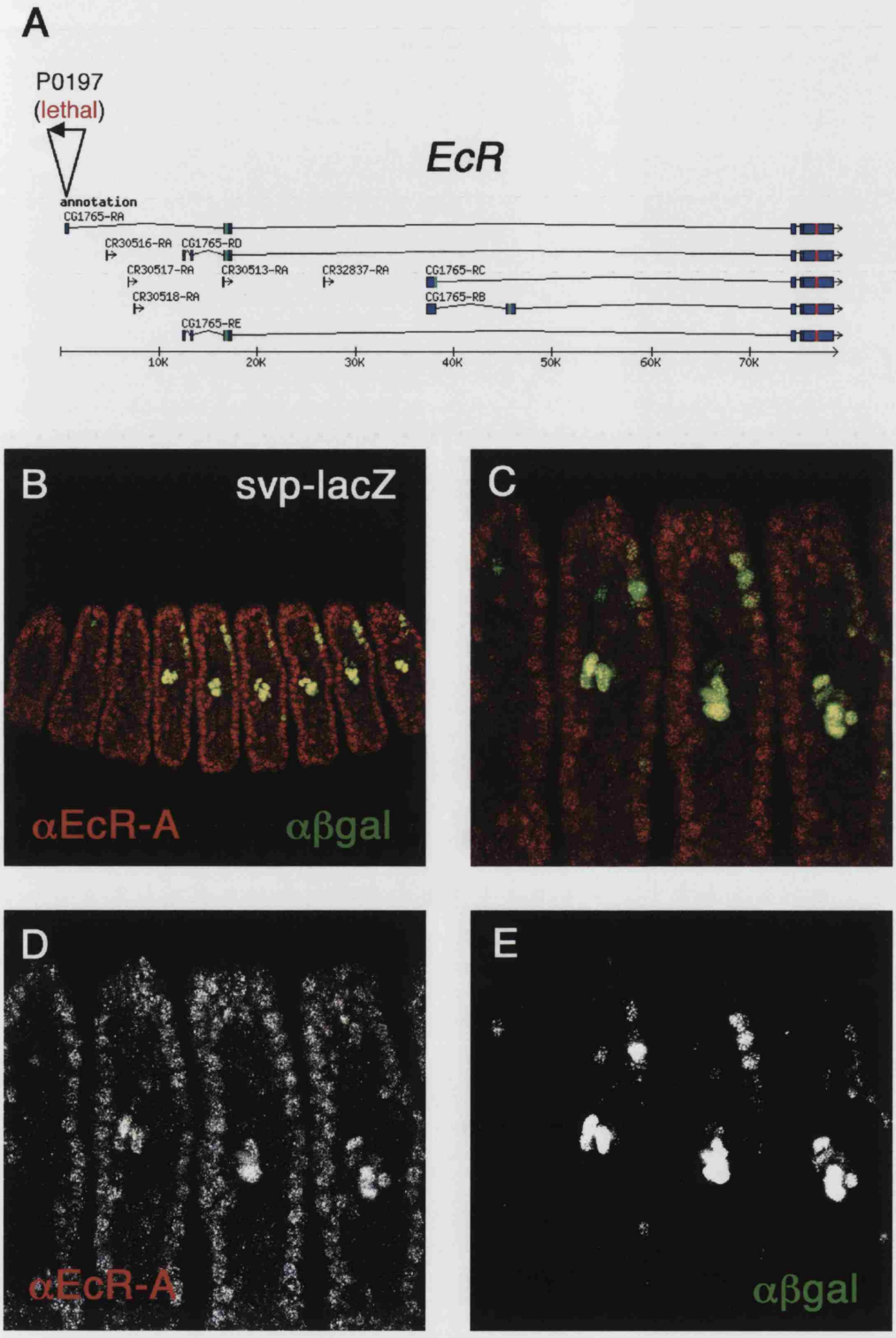
Eip75B protein isoforms are shown together with their annotated CG number.

Eip75B isoforms	Mouse Highest E Value	Mouse First Rev-Erb E Value
E75A CG8127-PC	PPAR alfa 3E-53	Rev-Erb beta 5E-43
E75B CG8127-PA	PPAR alfa 4E-38	Rev-Erb beta 5E-31
E75C CG8127-PB	PPAR alfa 4E-53	Rev-Erb beta 7E-43

Figure 4.5 The *Ecdysone Receptor (EcR)* Locus and Embryonic Expression Pattern of *EcR-A*

(A) Genomic map of *EcR* indicating the insertion point of the P0197 enhancer trap into the first non-coding exon of the A isoform transcript of *EcR*.

(B-D) A stage 13 *svp-lacZ[S10]* embryo immunolabelled with anti-ß-gal (green) and anti-EcR-A (red). EcR-A expression at this stage is observed in oenocytes and epidermal cells.


A
P0197
(lethal)
EcR
annotation
CG1765-RA
CR30516-RA
CG1765-RD
CR30517-RA
CR30513-RA
CR32837-RA
CG1765-RC
CR30518-RA
CG1765-RB
CG1765-RE
10K
20K
30K
40K
50K
60K
70K
B
svp-lacZ
αEcR-A
αβgal
C
D
αEcR-A
E
αβgal

a nuclear hormone receptor that plays a key role in steroid hormone-regulated developmental transitions. *EcR* has been placed as an immediate-early gene at the top of a genetic hierarchy responsive for ecdysone, leading to the activation of early and late genes (reviewed in (Riddiford 1993). Differential expression of *EcR* isoforms, within different tissues and times, have been suggested to be involved in the different metamorphic responses that these tissues display (Talbot, Swyryd et al. 1993; Truman, Talbot et al. 1994). Expression of isoform A within oenocytes was confirmed by immunocytochemistry using *svp-lacZ[S10]* as an oenocyte marker (Figure 4.5B to Figure 4.5E). A brief burst of high expression of *EcR-A* during embryonic stage 13 is detected in oenocytes, while the epidermis maintains moderate levels of expression throughout embryogenesis. Interestingly, the P0197 reporter specifically recapitulates the oenocyte but not the epidermal aspect of EcR-A expression. The precise role of *EcR*, and in particular, *EcR-A*, in oenocytes remains to be elucidated.

Hepatocyte nuclear factor 4 (Hnf4)

Three independent insertions were identified in the *Hepatocyte nuclear factor 4 (Hnf4)* locus (Figure 4.6A). Two viable traps (L14A2 P{*GFP*} and OK72 P{*GAL4*}) were inserted in an intron specific for the A transcript and upstream of the promoters of the B and C transcripts. One lethal insertion (l(2)k04003 P{*lacZ*}) was found to be inserted 1kb downstream of the 3' end of the *Hnf4* messages. During late stages of embryogenesis, *Hnf4* mRNA has been observed in the oenocytes, the midgut and the Malpighian tubules, with expression in fat body being controversial (Zhong, Sladek et al. 1993; Hoshizaki, Blackburn et al. 1994). None of the three *Hnf4* lines that I identified are expressed in oenocytes at embryonic stages (Table 4.1 and Figure 4.6B). However, time courses of *Hnf4* mRNA and protein expression both revealed that oenocyte expression begins at embryonic stage 13 and is maintained throughout the remainder of embryogenesis (Figure 4.6C-E). By L3, all three enhancer traps have become expressed in oenocytes (Table 4.1), possibly reading out a late activation of the endogenous gene that might be distinct from the embryonic phase of expression. Based on the expression pattern of *Hnf4*, together with the analysis of large chromosome deletions, this gene has been implicated in the development of Malpighian tubules, salivary glands and the endodermal component of the midgut (Zhong, Sladek et al. 1993). However, the role of *Hnf4* during oenocyte differentiation and function remains to be investigated. Interestingly, mouse studies

Figure 4.6 Genomic Map and Expression Pattern of *Hepatocyte Nuclear Factor 4 (Hnf4)*

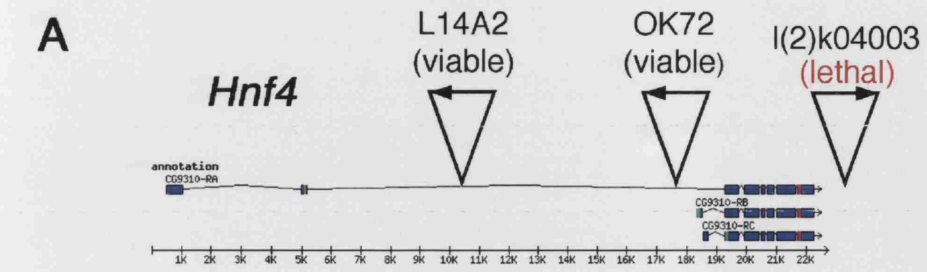
(A) Genomic map of *Hnf4* indicating the insertion points of three enhancer traps identified for this locus.

(B) A stage 15 *Hnf4[OK72] >lacZ* embryo immunolabelled with anti-ß-gal. Salivary glands show strong ß-gal expression. Oenocytes are not labelled at this stage.

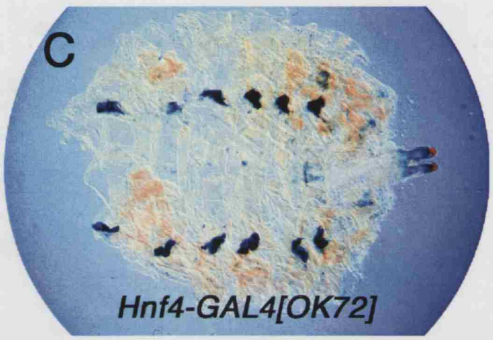
(C) Dissected pelt of a *Hnf4[OK72] >lacZ* L3 larva showing strong ß-gal expression in the oenocytes.

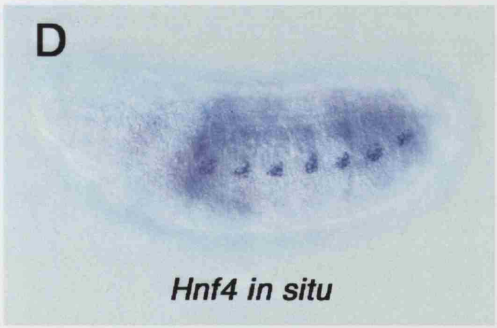
(D-E) *In situ* hybridization for *Hnf4* in a stage 15 embryo showing oenocyte expression.

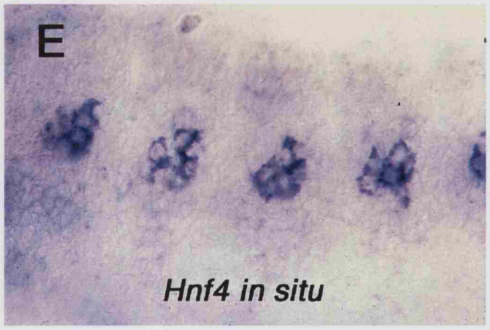
(F-H) Wild-type embryos immunolabelled with anti-Hnf4. (F) Stage 12 embryo showing Hnf4 expression in amnioserosa. (G) Confocal section at epidermal level of a stage 15 embryo showing expression in oenocytes. (H) Internal confocal section of same embryo as in (G) showing expression in foregut, hindgut, amnioserosa and Malpighian tubules (arrowheads).

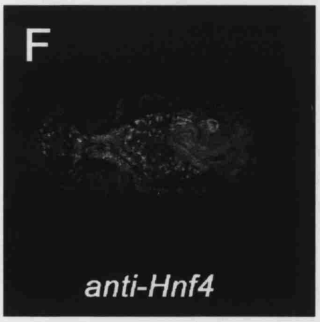

A
Hnf4
L14A2
(viable)
OK72
(viable)
l(2)k04003
(lethal)
annotation
CG9310-RA
CG9310-RB
CG9310-RC
1K 2K 3K 4K 5K 6K 7K 8K 9K 10K 11K 12K 13K 14K 15K 16K 17K 18K 19K 20K 21K 22K


B
Hnf4-GAL4[OK72]

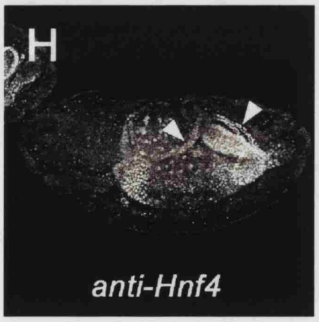

C
Hnf4-GAL4[OK72]


D
Hnf4 in situ


E
Hnf4 in situ


F
anti-Hnf4


G
anti-Hnf4


H
anti-Hnf4

suggest that *HNF-4α*, a close vertebrate relative, is essential for the development, differentiation and function of mammalian hepatocytes (Li, Ning et al. 2000; Parviz, Matullo et al. 2003).

seven-up (svp)

seven-up (svp) encodes an orphan nuclear hormone receptor, related to the vertebrate Chicken Ovalbumin Upstream Promoter-Transcripiton Factor (COUP-TF) family. At the start of this enhancer trap screen, a lethal P-insertion into *svp* (*svp[don1]*) was shown to be expressed in oenocytes (Elstob, Brodu et al. 2001). In addition, I found another lethal P-element insertion, l(3)A6-3-56[1], in the promoter region of the *svp* gene (Figure 4.7A). However, the lethality associated with this line may not be attributed to the *svp* P-element as a second insertion was detected in the essential locus *trithorax (trx*, see Table 4.1). The function of *svp* has been studied in the context of compound eye and Malpighian tubule development. In both cases, *svp* has been shown to act downstream of the *svp* EGFR signalling pathway (Begemann, Michon et al. 1995; Kerber, Fellert et al. 1998). In the eye, *svp* is required for development of four of the eight types of photoreceptor. Inaddition, *svp* is required for the regulation of cell divisions in proliferating Malpighian tubules. As with both of these examples, in oenocytes, *svp* expression lies downstream of EGFR activation (Elstob, Brodu et al. 2001) and *svp* mutants display an abnormal oenocyte migration phenotype (Gould 2000). Interestingly, *psq*, another gene found to be expressed in oenocytes (Section 4.3.6), has been identified as a *svp* target required for the development of photoreceptors R3 and R4 (Weber, Siegel et al. 1995), suggesting that a similar regulatory relationship might exist during oenocyte induction.

4.3.6 Other Transcription Factors

pipsqueak (psq)

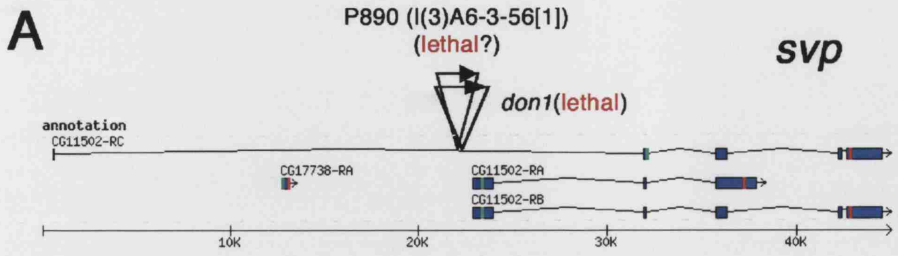
P0937, a lethal P{*lacZ*}, was inserted in the promoter region of transcripts *CG2368-RD* and *CG2368-RH* of *pipsqueak (psq)*, a gene encoding a nuclear BTB-containing protein (Figure 4.7B). This insertion is intronic with respect to the remaining five predicted *psq* transcripts. Embryonic and larval *lacZ* expression in the P0937 line is similar to the reported expression for *psq* transcripts and proteins at these stages (Table 4.1 and (Weber, Siegel et al. 1995). Interestingly, strong alleles of

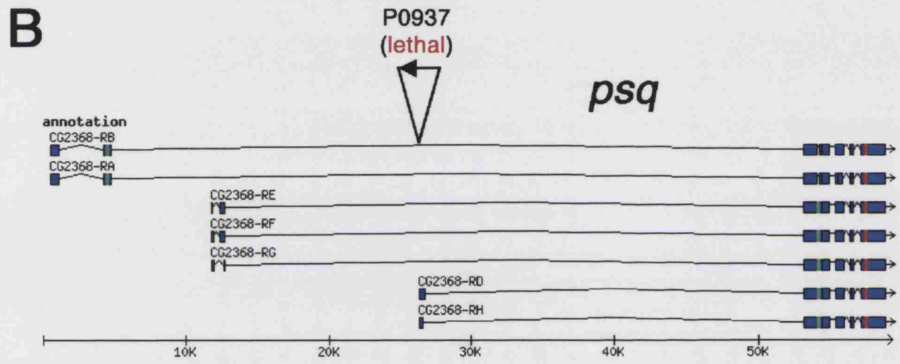
Figure 4.7 The *seven-up (svp), pipsqueak (psq), snail (sna)* and *Translocase Inner Membrane 17 (Tim17b2)* Loci

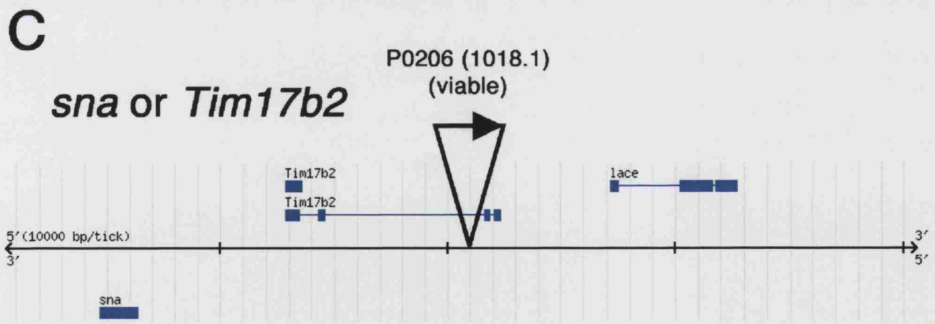
(A) Genetic locus for *svp* indicating the insertion of the l(3)A6-3-56[1] enhancer trap into the same promoter region was the previously characterised *don1* allele. Both alleles lie at the 5' end of *svp* isoforms *CG11502-RA* and *CG11502-RB.*

(B) Genomic locus of *psq* indicating the insertion of P0937 enhancer trap in the promoter region of *psq* isoforms *CG2368-RD* and *CG2368-RH.*

(C) Genomic region containing *sna*, *Tim17b2* and *lace*. The insertion of the P0206[1018.1] enhancer trap is indicated.


A
P890 (l(3)A6-3-56[1])
(lethal?)
svp
don1(lethal)
annotation
CG11502-RC
CG17738-RA
CG11502-RA
CG11502-RB
10K
20K
30K
40K


B
P0937
(lethal)
psq
annotation
CG2368-RB
CG2368-RA
CG2368-RE
CG2368-RF
CG2368-RG
CG2368-RD
CG2368-RH
10K
20K
30K
40K
50K


C
sna or Tim17b2
P0206 (1018.1)
(viable)
Tim17b2
Tim17b2
lace
5′(10000 bp/tick)
3′
3′
5′
sna

psq have been found to suppress a *svp*-induced transformation of cone cells within the eye (Weber, Siegel et al. 1995). As *seven-up (svp)* is also expressed in oenocytes, as an early-response to their induction (Elstob, Brodu et al. 2001; Brodu, Elstob et al. 2002), these results raise the possibility that *psq* may act downstream of *svp* during oenocyte induction by EGR receptor signalling.

snail (sna)* and *Translocase inner membrane 17 (Tim17b2)

A viable P{*GAL4*} insertion (P0206[1018.1]) with embryonic and larval expression in oenocytes was found to be inserted in an intronic sequence of *Translocase inner membrane 17 (Tim17b2)* and about 17kb upstream of *snail (sna)* (Figure 4.7C). *Tim17b2* encodes a product with protein translocase activity involved in protein-mitochondrial targeting (Sardiello, Licciulli et al. 2003). *snail (sna)* encodes a zinc-finger C2H2-type transcriptional repressor required for mesoderm formation. It is involved in the establishment of the mesoderm-neuroectoderm boundary by repressing expression of neuroectodermal genes (Kosman, Ip et al. 1991). In later stages of embryogenesis, *sna* is a regulator of neurogenesis, (Ip, Levine et al. 1994). As *sna* appears to be expressed in oenocytes from stage 11 to stage 13 (Ip, Levine et al. 1994; BDGP 2003), the P0206 P-element will be referred to here as *sna-GAL4*. However, the expression pattern of *Tim17b2* is unknown and further characterization of the *Tim17b2* and *sna* loci are required to define which of these two genes is regulating the enhancer trap insertion.

diminutive (dm)

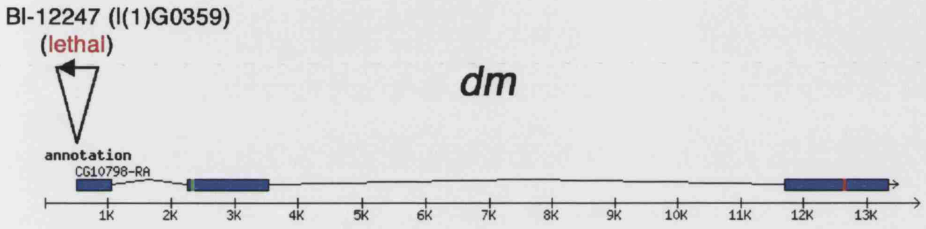
The lethal P insertion, l(1)G0359, is located within the promoter region of *diminutive (dm)* (Figure 4.8A). This *lacZ* enhancer trap is selectively expressed in embryonic oenocytes and a few other tissues (Table 4.1 and Figure 4.8B). During late embryonic stages *dm* mRNA is expressed in the anterior and posterior midgut primordium, lymph gland, some muscles and the hindgut (Gallant, Shiio et al. 1996; BDGP 2003). Expression of *dm* mRNA in oenocytes remains to be confirmed. *dm* is the *Drosophila* ortholog of the protooncogene *Myc* and studies in the wing have suggested that it links patterning to cell division by regulating primary targets involved in cellular growth and metabolism (Johnston, Prober et al. 1999). Even though oenocytes do not undergo cytokinesis during or after their induction (Brodu and Gould, in preparation), they become polyploid and increase greatly in size from

Figure 4.8 The *diminutive (dm)* Locus and Embryonic Expression Pattern of the l(1)G0359 Enhancer Trap

(A) Genomic map of *dm* indicating the insertion of l(1)G0359 into the promoter region.

(B) A stage 17 *l(1)G0359-lacZ* embryo immunolabelled with anti-ß-gal. Reporter expression is seen in oenocytes (dotted circle), some epidermal cells and gut (out of focal plane).

A


BI-12247 (l(1)G0359)
(lethal)
dm
annotation
CG10798-RA
1K
2K
3K
4K
5K
6K
7K
8K
9K
10K
11K
12K
13K


B
dm-lacZ[l(1)G0359]

~5μm diameter when they delaminate to an impressive size of ~80μm during third larval instar. This cell size increase is much greater than that of surrounding epidermal cells and might reflect a high demand for *dm* function.

hindsight (hnt)

The lethal element l(1)G0237 is inserted in the promoter region of *hindsight (hnt)*, a gene coding for a C2H2-type zinc finger transcription factor (Figure 4.9A). l(2)G0237 recapitulates Hnt protein expression in the oenocytes and PNS of embryos (Table 4.1, Figure 4.9B and Figure 4.10). In addition, oenocyte but not PNS expression of l(1)G0237 is maintained in L3 larvae (Figure 4.9C). *hnt* is known to be involved in several developmental processes, such as the maintenance of the amnioserosa, which is required for proper germ band retraction (Yip, Lamka et al. 1997) and dorsal closure (Reed, Wilk et al. 2001). *hnt* is also required for epithelial maintenance, differentiation of taenidia (Wilk, Reed et al. 2000), and multiple steps during ommatidial development (Pickup, Lamka et al. 2002). Expression of *hnt* was noted previously in oenocytes (Wilk, Reed et al. 2000), however its functions in this cell remain to be identified. To characterize further *hnt* oenocyte expression, an immunostaining time course was performed, using *svp-lacZ[don1]* embryos to label the oenocytes. At early stage 11, oenocyte precursors at the onset of delamination do not express Hnt (Figure 4.10A). Soon after however, the oenocyte whorls at late stage 11 do become positive for Hnt (Figure 4.10B). This oenocyte expression is maintained throughout the remainder of embryogenesis (Figure 4.10C-D). Double-staining of embryos for *hnt* and the neuronal marker ELAV provides an independent method to confirm *hnt* expression in the oenocytes (Figure 4.10E-F).

spalt (sal)

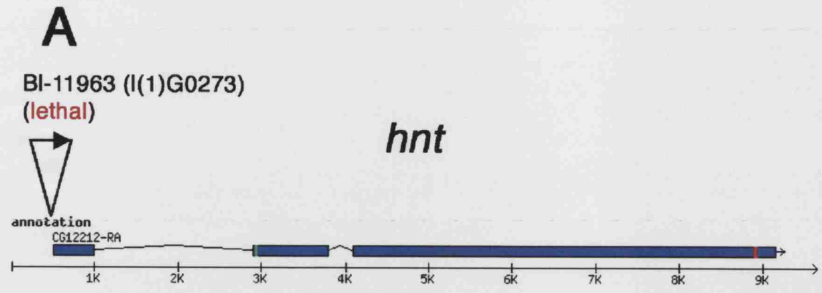
Two *lacZ* traps already identified as being inserted into the *spalt* complex l(2)03602 (P1340) and A405 (Wagner-Bernholz, Wilson et al. 1991; Kuhnlein, Frommer et al. 1994; BDGP Project Members 1994-1999), were found to be expressed in oenocytes (Figure 4.11A and Table 4.1). The *spalt (sal)* gene codes for a zinc-finger transcription factor with dual roles in priming oenocyte induction and as part of the early oenocyte-specific EGFR response as has been previously described (Introduction, Section 1.7.2). As the P1340 line retains oenocyte expression in L3, it may be that Sal also plays a later role in oenocyte function during larval stages.

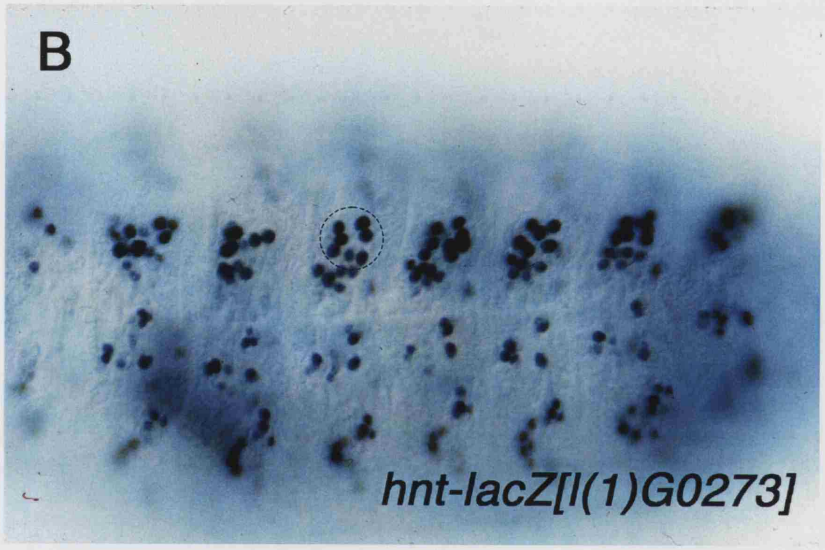
Figure 4.9 The *hindsight (hnt)* Locus and Expression Pattern of l(1)G0273 Enhancer Trap

(A) Genomic map of *hnt* indicating insertion of l(1)G0273 into the promoter region.

(B) A stage 17 *l(1)G0273-lacZ* embryo immunolabelled with anti-ß-gal showing expression in oenocytes (dotted circle) and peripheral neurons (Also see figure 4.9 and text for details).

(C) Detail of a dissected pelt of an L3 *l(1)G0273-lacZ* larva showing reporter expression in oenocytes.


A
BI-11963 (l(1)G0273)
(lethal)
hnt
annotation
CG12212-RA
1K
2K
3K
4K
5K
6K
7K
8K
9K


B
hnt-lacZ[l(1)G0273]

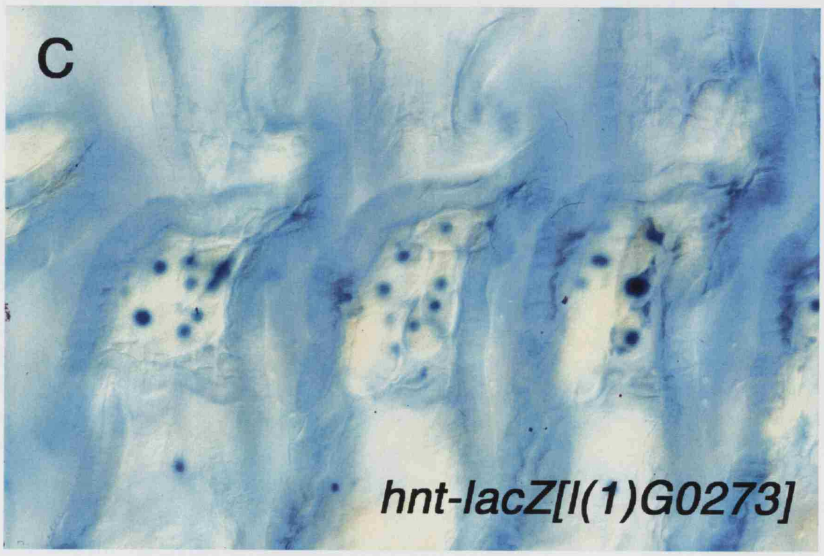

C
hnt-lacZ[l(1)G0273]

Figure 4.10 Time Course of *hnt* Embryonic Expression in Oenocytes and PNS

(A-D) Time course of Hnt expression during oenocyte development. *svp-lacZ* embryos of these panels were immunolabelled for Hindsight (red) and ß-gal (green).

(A) During early stage 11 *hnt* is not expressed in oenocyte precursors but these cells already express the *svp-lacZ* marker.

(B) A late stage 11 embryo showing co-localization of *svp-lacZ* expression and Hnt in oenocyte precursors.

(C-D) *hnt* expression is maintained in oenocytes during embryogenesis as seen in a stage 13 (C) and a stage 16 embryo (D).

(E-F) A stage 17 embryo immunolabelled against Hnt (red) and with ELAV antibody (green). Oenocytes can be distinguished from adjacent Hnt-positive peripheral neurons as the former do not express the ELAV neuronal marker. Arrowheads in (D) and (F) point to oenocyte cells, while the other cell types are neuronal.

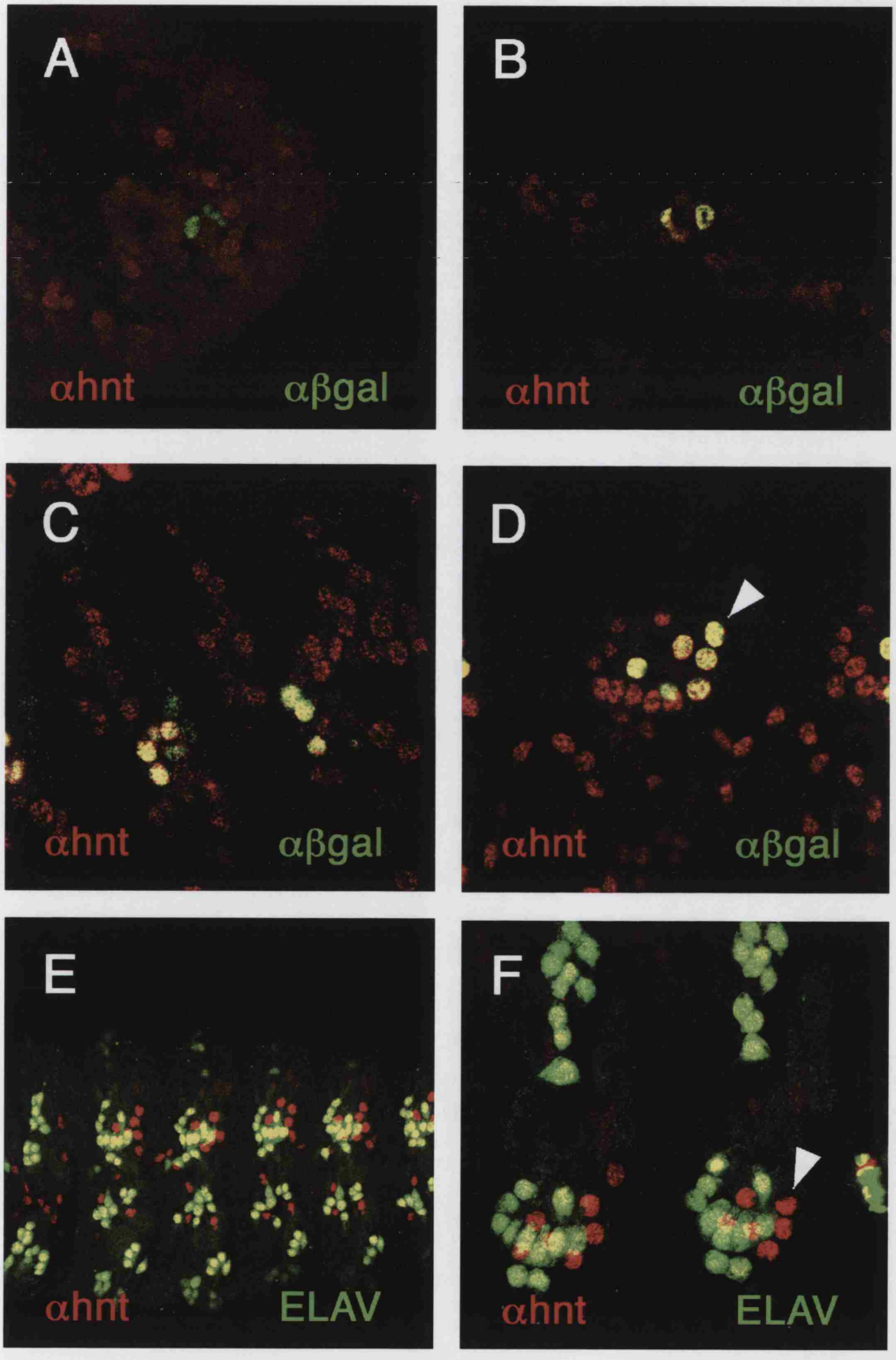
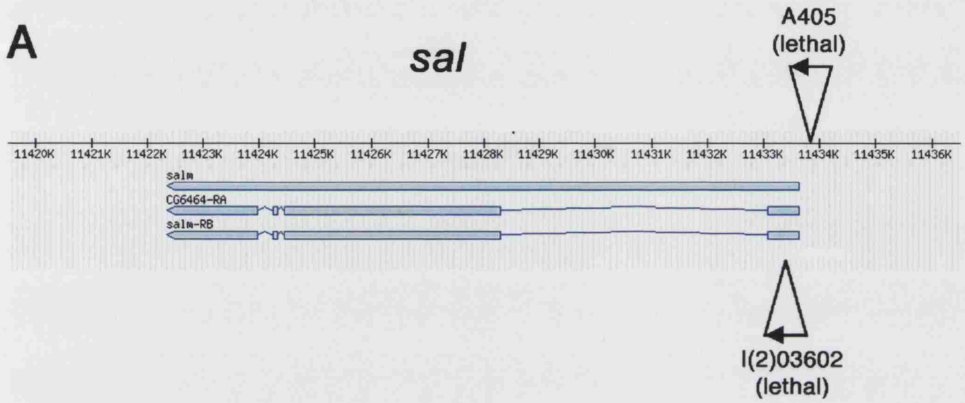
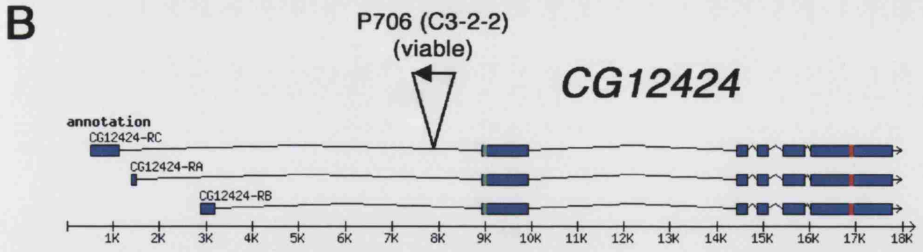

A
αhnt
αβgal
B
αhnt
αβgal
C
αhnt
αβgal
D
αhnt
αβgal
E
αhnt
ELAV
F
αhnt
ELAV

Figure 4.11 The *spalt(sal)* and *CG12424* Loci

(A) Genomic map for *spalt* indicating insertion points of A405 and l(2)03602 enhancer traps.

(B) Genomic map for *CG12424 (pnt-like)* indicating the insertion of the C3-2-2 enhancer trap.


A
sal
A405
(lethal)
11420K 11421K 11422K 11423K 11424K 11425K 11426K 11427K 11428K 11429K 11430K 11431K 11432K 11433K 11434K 11435K 11436K
salm
CG6464-RA
salm-RB
l(2)03602
(lethal)


B
P706 (C3-2-2)
(viable)
CG12424
annotation
CG12424-RC
CG12424-RA
CG12424-RB
1K 2K 3K 4K 5K 6K 7K 8K 9K 10K 11K 12K 13K 14K 15K 16K 17K 18K

***CG12424* (a pnt-like TF)**

A viable P{*lacZ*} insertion (C3-2-2) was detected in an intron common for all isoforms of the predicted gene *CG12424* (Figure 4.11B). Interestingly, the product of this novel gene is a transcription factor containing a *pointed (pnt)* domain. *pnt* encodes a ETS-domain transcription factor, target of the Ras/MAP kinase pathway that is known to act downstream of the EGFR (Scholz, Deatrick et al. 1993; O'Neill, Rebay et al. 1994). *Pnt* is known to be expressed in oenocytes (Elstob, Brodu et al. 2001; Brodu, Elstob et al. 2002) suggesting that both of these genes may play complementary or overlapping roles in the oenocyte response to EGFR signalling. *CG12424* expression and function has yet to be analysed.

C2H2-type Zn-finger TF (CG4427)

A viable P{*GAL4*} (56B) was inserted 5' of the predicted transcript of *CG4427* transcript A and 1kb 5' of transcript B (Figure 4.12A). *CG4427* codes for a predicted C2H2-type zinc finger transcription factor. Overexpression of a P{*EP*} element 228bp 5' of the predicted ATG start codon of this gene leads to defects in synaptogenesis and sensory organ formation, including enhancement of the *pannier (pnr)* phenotype (Abdelilah-Seyfried, Chan et al. 2000; Kraut, Menon et al. 2001; Pena-Rangel, Rodriguez et al. 2002). A comparison between the embryonic expression of *CG4427* mRNA and that of the *GAL4[56B]* reporter revealed similar expression patterns in the amnioserosa and dorsalmost epidermal cells neighbouring it (Figure 4.12B and Figure 4.12C). This confirms the identity of the gene trapped. The role of *CG4427* in oenocytes, where it is likely to be expressed during larval but not embryonic stages (Table 4.1), remains to be elucidated.

4.3.7 Miscellaneous Proteins

ß-galactosidase (CG3132)

CG3132 predicted product was homologous to vertebrate lysosomal ß-galactosidases known to degrade the carbohydrate component of lipoproteins, a highly characterised group of enzymes involved in carbohydrate transport and metabolism. During embryogenesis, *CG3132* is expressed in oenocytes, midline, dorsal vessel and circulatory system, optic lobe and head epidermis (See Appendix 3).

Figure 4.12 Genomic Map and Expression Pattern of *CG4427*, Encoding a C2H2 Zinc-Finger Transcription Factor

(A) Genomic map for *CG4427* indicating the insertion of the 56B enhancer trap 5' of *CG4427-RA*.

(B) Dorso-lateral view of a stage 13*GAL4[56B];UAS--lacZ* embryo immunolabelled for ß-gal showing reporter expression in the amnioserosa and some very epidermal cells.

(C) Endogenous expression of *CG4427* mRNA in a dorso-lateral view of another stage 13 embryo, showing expression pattern similar to that of the 56B reporter gene.

A


56B
(viable)
annotation
CG4427
CG4427-RA
CG4427-RB
1K
2K
3K
4K

B


56B>lacZ

C


CG4427 in situ

Recently, two genome-wide studies have identified *CG3132* as a gene upregulated during programmed cell death (Gorski, Chittaranjan et al. 2003; Lee, Clough et al. 2003).

amnesiac (amn)

Four independent viable insertions (P030, P0093, P0110 and P0103) were all identified in the monoexonic *amnesiac (amn)* gene (Figure 4.13). *amn* encodes a neuropeptide precursor with similarities to mammalian growth-hormone-releasing hormone (GHRH) and pituitary adenylyl cyclase-activating peptide precursor (PACAP, (Feany and Quinn 1995; Moore, DeZazzo et al. 1998). In agreement with the viablility of the P-insertions, it has been reported that a small deletion removing the *amn* open-reading frame is viable (Moore, DeZazzo et al. 1998). *amn* expression in the dorsal bipolar neurons (DPNs) has been associated with learning and memory via the regulation of calcium oscillations in the mushroom bodies (review by (Davis 2001). a*mn* has also been linked to ethanol sensitivity, but this effect is not due to its expression in the mushroom bodies (Moore, DeZazzo et al. 1998), raising the possibility that oenocytes might be involved in ethanol metabolism during larval stages. However, as I have tried, thus far without success, to detect *amn* transcripts and protein in oenocytes, it may be that enhancer-trap expression in oenocytes does not reflect the true *amn* expression pattern. Moreover, the entire *amn* locus is contained inside the intron of another predicted gene, *CG32529* (Figure 4.13). The predicted product of *CG32529* has no homology to any other protein in the database and its expression remains to be determined.

Na+ H+ exchanger 2 (NHE2)

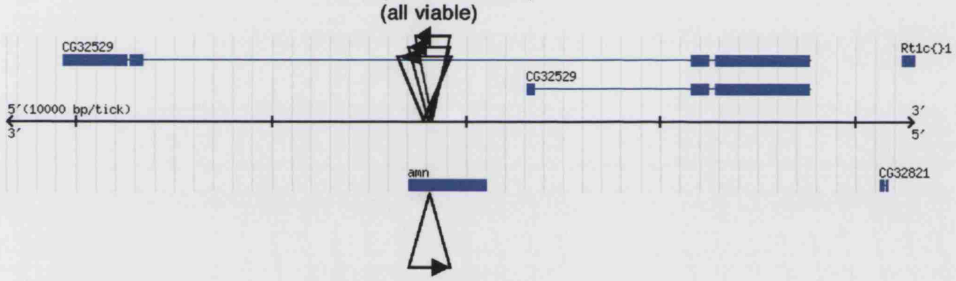
NHE2 codes for a sodium-hydrogen exchanger (Giannakou and Dow 2001). NHEs are involved in intracellular pH homeostasis, cell volume control, and electroneutral NaCl absorption in epithelia (reviewed in (Yun, Tse et al. 1995). There are two other fly members of this family of transporters and transcripts for all three fly *NHEs* have been detected in Malpighian tubules (Giannakou and Dow 2001). The precise function of *NHE2* within the oenocyte context remains to be addressed.

Figure 4.13 The *amnesiac (amn)* Locus

Four independent enhancer traps are inserted into a hot spot in *amn* coding region. The entire *amn* locus is contained within an intron of one of C*G32529* isoforms.

amn

P030 (SW084-A7.10XW)
P0093 (MW057-130a)
P0110(ES074-69.35.2)
(all viable)



P0103 (ES012-65.12.1)
(viable)

MSP-300/nesprin (CG31649)

Muscle-specific protein 300 (Msp-300)/nesprin encodes a gigantic actin-binding protein. This gene has two independent promoters that can encode completely different isoforms (MSP-300 and nesprin). In addition, the last exons of *Msp-300* can be spliced onto exons of adjacent *nesprin* to form MSP-300/nesprin, a hybrid protein of around 11,270 residues (Zhang, Ragnauth et al. 2002). *Msp-300* and *nesprin* are both expressed at muscle attachment sites and disruption of *Msp-300* results in muscle defects (Volk 1992).

Immunoglobulin domain-containing protein (CG31361)

CG31361 encodes a protein with two immunoglobulin-like domains. Its product shares only low-level similarity with other proteins of the immunoglobulin superfamily and its possible function has yet to be tested.

CG3328* and *CG31764

BLASTP searches for two oenocyte genes found from the BDGP *in situ* site (*CG3328* and *CG31764*) failed to find significant similarity to any known protein motif. Nevertheless, in both cases, matches are found to predicted proteins from various eukaryotes, including humans, indicating that the products of these novel genes are highly conserved and probably share functionality.

4.3.8 Comparison of Screening Methods

From the enhancer-trap and *in situ* approaches used to identify oenocyte genes, only one gene (*CG11151*) was found by both methods. This is probably because neither screen has reached saturation. In this regard, enhancer traps and mRNAs expressed in oenocytes may have been overlooked as appears to be the case with *amn-GAL4[28A]* where embryonic staining of the oenocytes was reported to be in peripheral neurons (DeZazzo, Xia et al. 1999). A similar mix up occurred when the *sna* pattern of expression was described (Ip, Levine et al. 1994). Perhaps more significantly, the present BDGP *in situ* database comprises only 1711 genes, just over 10% of the predicted genes in release 3 of the genome. A new resource that promises to help to expand the number of oenocyte enhancer-traps is the *GAL4*-Enhancer Trap DataBase (GETDB), recently developed in Japan (Hayashi, Ito et al. 2002). This database combines expression patterns for all stages of the fly life cycle with insertion site information for 7000 independent P-GAL4 insertions. Preliminary searches for lines

expressed in oenocytes revealed 344 lines, of which 120 are expressed in embryonic stages, while 224 are expressed at larval stages. Together these data suggest that the total number of genes having a restricted expression pattern including the oenocytes is several hundred. Although in this thesis only a small fraction of this total is described, this is sufficient to provide a scaffold of the biochemical pathways and regulatory networks upon which additional oenocyte genes can be placed.

4.4 Testing the Roles of Individual Genes in Oenocyte Differentiation

Having identified a set of 35 oenocyte genes, I have recently begun addressing the requirement of individual genes in oenocyte differentiation and function. In this section, these two approaches will be described.

4.4.1 Regulation of *Alas* by Other Oenocyte Genes

I have begun to position some of the genes identified by enhancer trapping within a genetic hierarchy of oenocyte differentiation. A detailed analysis of the expression profile of 7 oenocyte genes led to their classification according to the time at which they are expressed, relative to oenocyte induction at stage 11 (Figure 4.14A). *sal, hnt* and *svp* were grouped as immediate-early genes, *Hnf4* and *EcR-A* as early genes, while *CG11151* and *∂-Aminolevulinate synthase (Alas)* were considered as late genes.

Alas encodes for a mitochondrial enzyme involved in the synthesis of the widely used heme prosthetic group. Alas catalyses the first and rate-limiting step in the heme biosynthetic pathway: the reaction of glycine with succinyl-CoA to produce ∂-aminolevulinate (reviewed in (Ryter and Tyrrell 2000). In vertebrates, Alas is encoded by two different genes. One of them, *Alas2* or *AlasE,* is exclusively expressed in erythroid cells, where heme is required for the synthesis of hemoglobin. The second gene, *Alas1 or AlasN,* is a housekeeping gene expressed in all cell types, where heme is attached to cytochromes and other hemoproteins, such as Catalase (reviewed in (Ryter and Tyrrell 2000). *Drosophila* lacks hemoglobin and contains only one *Alas* gene, sharing the highest similarities to the vertebrate housekeeping isoform. In late embryonic stages, it is specifically expressed in oenocytes, suggesting a role of these cells in the biosynthesis of hemoproteins, such as catalase and cytochrome P450s such as Cyp4g1 (Ruiz de Mena, Fernandez-Moreno et al. 1999).

These findings suggested that *Alas* could be a key oenocyte gene expressed during the late stages of oenocyte differentiationand being involved in oenocyte

Figure 4.14 *sal, hnt, EcR* and a Deficieny Removing *Hnf4* Are Required for the Formation of *Alas*-Positive Oenocytes

(A) Time windows of embryonic expression of seven oenocyte-genes.

(B-G) All panels show embryos *in situ* hybridised for *Alas*. All panels except (E) are stage 16-17 embryos.

(B) A wild-type embryo showing normal *Alas* expression in all 7 oenocyte clusters.

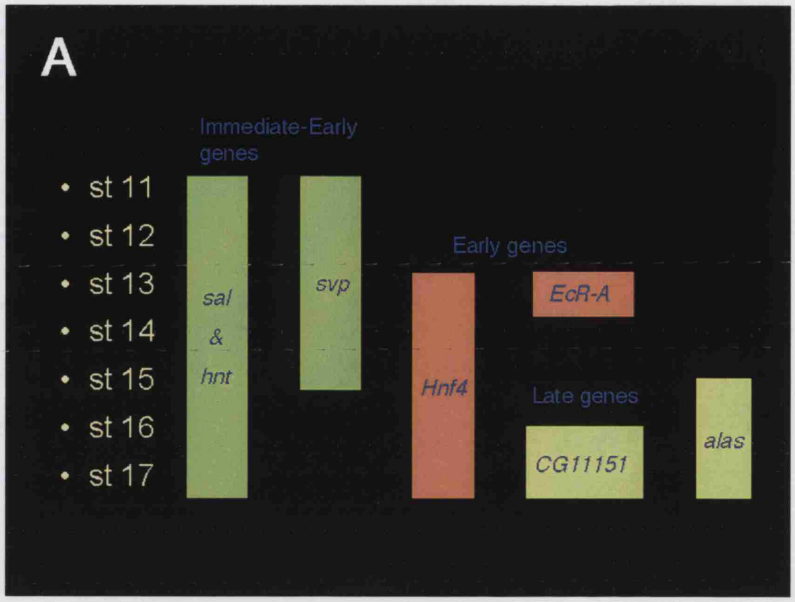
(C) *sal[16]* null mutant embryo shows loss of *Alas*-positive oenocytes except in the occasional oenocyte cluster (arrowhead).

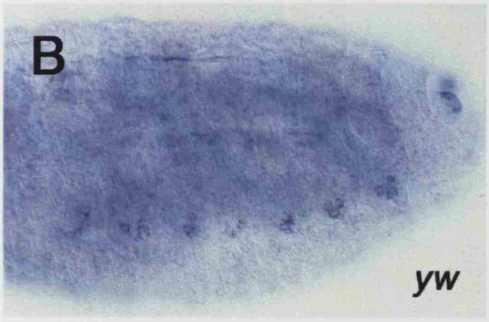
(D) A *svp[E22]* null mutant embryo, showing that dorsally misplaced oenocytes (arrowhead) retain *Alas* expression.

(E) A *hnt* null mutant embryo, identified by its abnormal unretracted germband, showing complete loss of *Alas*–positive oenocytes.

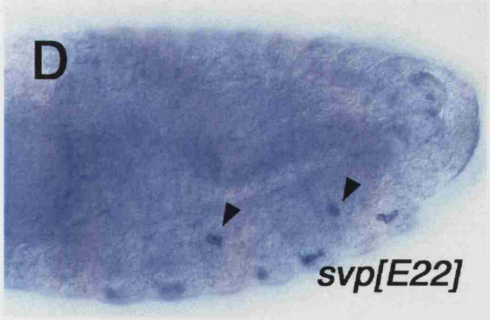
(F) A *EcR[V559fs]* null embryo lacking *Alas*-positive oenocytes in A1 and A6 (arrowheads)

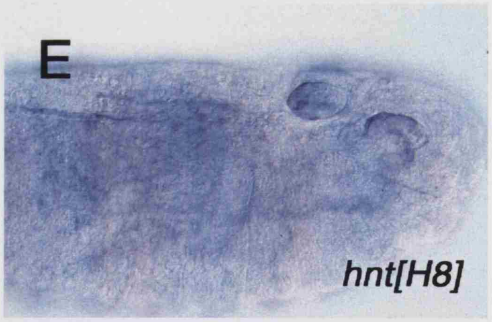
(G) An embryo homozygous for *Df(2L)N22-5,* a large deficiency uncovering the *Hnf4* locus, lacks all *Alas*-positive oenocyte clusters.

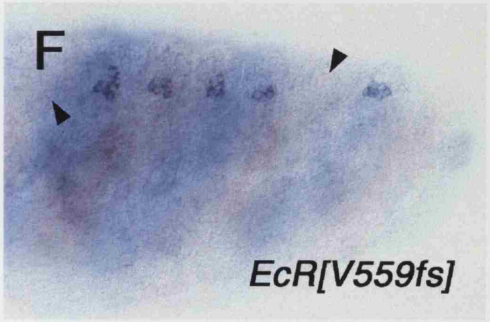

A
Immediate-Early genes
• st 11
• st 12
• st 13
• st 14
• st 15
• st 16
• st 17
sal & hnt
svp
Early genes
Hnf4
EcR-A
Late genes
CG11151
alas


B
yw


C
sal[16]


D
svp[E22]


E
hnt[H8]


F
EcR[V559fs]


G
Df(2L)N22-5

function. Therefore, I chose to monitor *Alas* expression in embryos mutant for immediate-early and early genes. Oenocytes of wildtype embryos begin *Alas* expression at embryonic stage 15 (Figure 4.14B). *sal* mutants are known to lack most oenocyte clusters (Elstob, Brodu et al. 2001) and consistent with this, most segments show no oenocyte-specific *Alas* expression. However, in those rare cases where a cluster is formed, it is *Alas*-positive, suggesting that there is unlikely to be a late role for Sal in activating Alas expression (Figure 4.14C). *svp* mutants have been reported to display defects in oenocyte migration (Gould 2000; Elstob 2001) but I find that *Alas* expression is normal even in aberrantly positioned oenocytes (Figure 4.14D). Embryos mutant for *EcR* exhibit the absence of a minority of oenocyte clusters, suggestive of *EcR* playing a partially redundant role with other nuclear hormone receptor genes (Figure 4.14F). More interestingly, *hnt*-null embryos, identified by the characteristic U-shape of an unretracted germband, displayed a complete loss of *Alas*-positive oenocytes (Figure 4.14E). This indicates either that oenocyte formation is blocked in *hnt* mutants or that, once induced they do not differentiate properly and thus fail to express *Alas*. Like *hnt* mutants, embryos homozygous for a deficiency uncovering the *Hnf4* locus exhibit the complete absence of *Alas*-positive oenocytes (Figure 4.14G). Interestingly, previous analysis of the upstream regulatory region of *alas*, has revealed potential binding sites for *Hnf4*, raising the possibility that *Hnf4>Alas* regulation is direct (Ruiz de Mena, Fernandez-Moreno et al. 1999). Together, these mutant studies suggest that while the immediate-early *svp* gene does not affect *Alas* expression, other immediate-early genes (*sal* and *hnt*) and some early genes (*EcR* and *Hnf4*) do. Further experiments are required to discriminate clearly whether these four genes act at the level of oenocyte induction or during later stages of oenocyte differentiation.

4.4.2 Oenocyte-specific gene knockdowns by an RNAi approach

The identity of 35 genes expressed in oenocytes has provided some important clues about the potential function of the oenocytes, which I address in the final discussion chapter. However, a more direct way of using these genes to ascertain oenocyte function involves a functional strategy. Several reverse-genetic approaches can now be used to generate mutant alleles in a given gene by excising sequence-verified P-insertions or using a TILLING approach (McCallum, Comai et al. 2000), targeting

deletions by homologous recombination or by using dominant-negative proteins or RNA knockdowns.

Some of the genes isolated such as *Cyp4g1* (*CG3972*), *Elongase* (*CG18609*), *3-HSD-like* (*CG17562*) and *Acyl-CoA N-acyltransferase* (*CG14615*) appear to be expressed exclusively or almost so in oenoctes. For this class of genes, the generation of null alleles may reveal directly their function in oenocytes. However, the majority of the oenocyte genes isolated are also expressed in other tissues, frequently the gut or the CNS. The direct study of alleles for this latter class of genes would reveal phenotypes caused by the combination of defects produced in many cell types, not necessarily just oenocytes.

Therefore, to address the requirement for both classes of genes within oenocytes, a cell-specific approach is required. For tissues undergoing cell division in larvae, approaches involving the use of FRT clones can be considered. However, in the case of oenocytes, none of these approaches is feasible, as there is no cell division following their initial induction (Section 1.7.2). Hence, to study the role of particular genes specifically in oenocytes, I decided to use an approach based on RNA interference (RNAi). I used a recently developedplasmid, *pWIZ*, in which inverted repeats can easily be cloned into a *pUAST*-derived transformation vector (Lee and Carthew 2003). Two properties of this vector are noteworthy; first, the presence of GAL4 binding sites enables a spatiotemporal control over the induction of RNAi and second, the presence of a intron spacer facilitates the cloning and stable maintenance of the inverted repeats in bacteria, while its splicing-out in flies is believed to produce more efficient mRNA knockdown than other approaches which use inverted repeats separated by a hairpin.

Five oenocyte-genes were selected for generation of *pWIZ-RNAi* constructs. This selection was based on the strength and specificity of expression in oenocytes, as well as a focus on lipid-metabolic and nuclear receptor genes. The genes selected were *Hnf4, svp, CG11151, Cyp4g1* and *CG18609*. Mutations in only one of these genes, *svp*, have previously been studied in detail. *Hnf4* and *svp* code for two orphan nuclear-hormone receptors that are closely related and thus might have overlapping functions. The other three members selected, *CG11151, Cyp4g1* and *CG18609*, represent genes predicted from the genome project but yet to be verified. *CG11151* encodes SCP2 and is expressed in the oenocytes and the gut, *Cyp4g1* is a cytochrome P450 gene, and *CG18609* codes for an elongation enzyme involved in synthesising

long-chain FAs. Importantly, both *Cyp4g1* and *CG18609* are expressed exclusively in oenocytes.

DNA fragments were amplified by PCR from cDNA clones representing each of the five genes. The resulting products ranged from 214bp-720bp in lenght. These fragments were cloned as inverted repeats into *pWIZ*, 20 independent lines were generated for each construct and their insertions mapped to particular chromosomes. At present, the efficiency of these lines for *UAS-RNAi* knockdowns are being tested.

4.5 Conclusion

Together, the results of the enhancer trap screen and the *in silico* screen, led to the identification of 35 genes expressed in oenocytes (Table 4.2). Strikingly, a high fraction of these (17 genes) encode proteins involved in the capture of lipoprotein complexes, their degradation, initial FA modification and subsequent degradation by ß-oxidation. In addition, four genes code for nuclear hormone receptors, raising the possibility that some of the resultant lipid metabolites may act as ligands for these transcription factors. In addition, expression of 8 more transcription factors, many of which are involved in the initial steps of oenocyte induction and differentiation, were also identified. The significance of these findings will be discussed in the next chapter.

I have begun to disentangle the function of these genes in oenocytes from their roles elsewhere in the body. First, I have begun to test their potential roles upstream of a late differentiation marker for oenocytes (*Alas*). In addition, RNAi vectors for five of the genes have been engineered to facilitate cell-specific gene inactivation.

CHAPTER FIVE

Discussion

"A worm tells summer better than the clock,
The slug's a living calendar of days;
What shall it tell me if a timeless insect
Says the world wears away?"

Dylan Thomas, 1933.

CHAPTER FIVE: Discussion

5.1 Ablation of Oenocytes Leads to a Strong Moulting Phenotype

This study has been focussed on elucidating the *in vivo* function of the larval oenocytes of *Drosophila*. To achieve this aim two approaches were used: ablation of oenocytes and identification of oenocyte-specific genes. The ablation approach used the *GAL4 x UAS-rpr* system to kill oenocytes during late embryonic stages. Refinement of the *GAL4/UAS-rpr* system included testing of different responder lines, to obtain the maximum degree of oenocyte deletion, as well as the detailed analysis of several enhancer elements, in search of the one that had the most restricted expression pattern and was thus most suitable as a GAL4 driver. The experiments described in Section 3.2, indicated that ablation of the oenocytes from embryonic stage 13 does not affect the development of two neighbouring tissues, the embryonic chordotonal organs and tracheal system. However, these studies did not address whether oenocytes play an earlier role in embryonic patterning, as there is a window of several hours from the moment when they are induced (stage 11) to the time when cell death is first activated. This possibility is suggested by the phenotypes of several mutations affecting oenocyte induction, which concomitantly produce chordotonal organ misplacements (Elstob, Brodu et al. 2001).

More conclusively, I have shown that the selective ablation of oenocytes produces larval arrest and an associated series of moulting defects. In particular, oenocyte-less larvae are asynchronous in their development, remain arrested during L2 or L3 stages and the majority die during the L2/L3 moult. Many of the persistent L3 arrested larvae display signs of having undergone a defective moult, as seen the duplication of several exoskeletal structures such as the mouth hooks, the cuticle and the trachae. As mentioned in Section 1.6.2, mutations affecting various components of the ecdysteroid signalling pathway are known to produce strikingly similar moulting phenotypes, suggesting that this pathway may be implicated in oenocyte function. The possible mechanisms by which oenocytes may control moulting and the molecules involved will be discussed in the next section.

5.2 Oenocytes Express Lipid-Processing and Regulatory Genes Characteristic of Hepatocytes

In vertebrates, the liver plays a central role in coordinating lipid metabolism and energy homeostasis (Section 1.2.2). In insects, not so much is known about the division of lipid metabolism by different tissue compartments. This study has shown that many lipid-metabolic genes are selectively expressed at high levels by oenocytes, strongly suggesting that this cell-type is specialised in processing lipids. Such a role for oenocytes would be consistent with their high density of smooth-ER and thus a lipid-rich structure. I will now argue that there are striking similarities between the genes expressed in oenocytes and those of vertebrate hepatocytes. I will illustrate how these similarities extend to various levels including: molecules involved in hepatocyte and oenocyte differentiation, molecules directly implicated in lipid degradation, and molecules regulating lipid metabolism.

5.2.1 Four Nuclear Hormone Receptors May Control Oenocyte Differentiation and Function

Four nuclear hormone receptors were identified as oenocyte-specific genes: *Hnf4α, EcR-A, svp, and Eip75B* in oenocytes. Importantly, vertebrate orthologs of all of these genes are expressed in hepatocytes, and perform crucial roles there. Nuclear receptors can act by forming heterodimers with other family members. Therefore, it is likely that oenocyte-specific nuclear receptors do not act independently but in different homo- and heterocombinations, varying according to particular developmental stages. Consistent with this, studies in hepatocytes suggest that all four classes of the nuclear receptors found in oenocytes *(Hnf4s, LXR, COUP-TFs* and *PPARs)* directly regulate the promoters of target genes forming different combinations of heterodimers (reviewed in (Waxman 1996; Tsai and Tsai 1997).

The murine equivalent of *Hnf4, Hnf4α,* is required both for hepatocyte development and function (Li, Ning et al. 2000; Parviz, Matullo et al. 2003). *Hnf4α-/-* embryos, if tetraploid rescued for the Hnf4α–early requirement in the visceral endoderm, show normal specification and early development of the liver primordium. However, in such partially-rescued embryos, hepatocytes fail to express a large set of late-differentiation genes, including those encoding apolipoproteins (*apoA1, apoAII, apoB, apoCIII* and *apoCII*), metabolic proteins (*aldolase B, phenylalanine hydroxylase*), serum factors (*albumin, transferring, retinol-binding protein and*

erythropoietin) and transcription factors (*HNF-1α* and *PXR*). Those results have led to the proposal that *Hnf4α* regulates serum cholesterol levels by controlling apolipoprotein expression and PXR expression, thus indirectly influencing cytochrome P-450 roles in thehydroxylation of steroid hormones and other substrates (Li, Ning et al. 2000). Along the same lines, targeted deletion of *Hnf4α* in fetal liver hepatocytes by means of the *Cre/loxP* system disrupts liver morphogenesis and leads to altered glucose metabolism (Parviz, Matullo et al. 2003). More specifically, hepatocytes fail to accumulate glycogen and the gluconeogenic enzymes Gys2, Pck1 and G6pc are all downregulated.

The closest vertebrate relative of *EcR*, the *Liver X Receptor (LXR)*, is believe to manage excess dietary cholesterol by regulating its catabolism (Kliewer, Lehmann et al. 1999). Two cholesterol metabolites, 24(S)-hydroxycholesterol and 24(S), 25-epoxycholesterol can act as ligands for LXRα, which when heterodimerised with RXR, activates transcription of the *7α-hydroxylase (CYP7a)* gene (Janowski, Willy et al. 1996; Lehmann, Kliewer et al. 1997; Peet, Turley et al. 1998). Cyp7a then catalyses the rate-limiting step in the conversion of cholesterol to bile acids which can then be excreted from the body.

Studies of the vertebrate *svp* orthologs, murine *COUP-TFI* and *COUP-TFII*, have suggested that COUP-TFs influence cell growth and cell differentation in the liver, through the regulation of the *Na+/H+ exchanger (NHE)* gene, a relative of another oenocyte gene (*dNHE2*, (Fernandez-Rachubinski and Fliegel 2001). Moreover, analysis of the *NHE1* promoter in liver nuclear extracts has shown that both COUP-TFs can activate transcription of this gene.

Finally, the orthologs of *Eip75B*, the vertebrate *PPARs*, orchestrate the regulation of lipid metabolism in the liver as described in the Introduction (see Section 1.4). The important implications of this will be addressed in depth in Section 5.2.3.

5.2.2 17 Oenocytes Proteins Have Predicted Roles in Lipid Degradation

At least 17 of the 35 oenocyte genes identified in this study encode proteins predicted to be involved in lipid metabolism. These genes code for two molecules involved in the capture of lipid-laden macromolecular complexes from the hemolymph (*LpR1 and LpR2*), 5 microsomal enzymes involved in FA modifications (*FALDH, Cyp4g1, Cpr, and elongases CG18609 and CG6921*), one putative peroxisomal transporter (*Atet*), 5

peroxisomal enzymes (*CG17562-3ß-HSD, CG12428-COT, CG9527-pristanoyl-CoA oxidase, Cat and CG11151-SCP2*) and one mitochondrial enzyme (*CG12262-Acyl-CoA-dehydrogenase*) and two other lipid enzymes whose precise position in a biochemical pathway is yet to be established (*CG7920-4-hydroxybutyrate CoA-transferase* and *CG12252 Acyl-CoA-N-acyltransferase*). Of the seven peroxisomal and mitochondrial proteins, at least five are involved in FA ß-oxidation. Taken together, the predicted functions and expression patterns of all these lipid-metabolic genes suggest that oenocytes possess all the machinery required for lipid degradation, from the elongation and ω-hydroxylation of FAs right through to their degradation via branches of the β-oxidation pathway in both peroxisomes and mitochondria.

5.2.3 Eip75 and Cyp4g1 Expression in Oenocytes May Regulate Larval Progression

Eip75B was isolated from the oenocyte enhancer trap screen as a gene expressed in larval but not embryonic oenocytes. The possibility that this *PPAR*-like gene is being expressed in oenocytes is particularly interesting because of the finding that several genes, related to known vertebrate PPAR targets involved in lipoprotein capture and lipid catabolism, are also expressed in oenocytes. This is the case for the six genes coding for a predicted P450 Cyp4 enzyme (*Cyp4g1*), a peroxisomal ABC transporter (*Atet*), a carnitine octanoyl transferase (*CG12428-COT*), catalase, an acyl-CoA oxidase (*CG9527-pristanoyl-CoA oxidase*), SCP2/SCPx (*CG11151-SCP2*) and medium-chain acyl-CoA dehydrogenase (*CG12262*, see Section 1.4.1). Although, at present, it has not been shown whether any of these oenocyte genes lie downstream of *Eip75B*, future analysis of *Eip75B* mutants will test directly *in vivo* whether the hepatocyte regulatory relationships are also present in oenocytes.

As cited in Section 1.4, it has been suggested that members of the Cyp4 family produce endogenous ligands for vertebrate PPARs. Candidate ligands so far described include metabolites of polyunsaturated FAs, derived from arachidonic acid. However, no arachidonates, or their precursors, have been found so far in analyses of *Drosophila* lipids (Section 1.5.2). The finding that a cytochrome P450 Cyp4 enzyme gene (*Cyp4g1*) and an LCFA-elongase gene (*CG18609*) are exclusively expressed in oenocytes, together with the fact that another LCFA-elongase gene (*CG6921*) also has a very restricted, oenocyte-inclusive, expression pattern, raises the possibility that

Eip75B ligands, similar to their vertebrate counterparts, are generated by the action of elongases and Cyp4 enzymes. Unlike the vertebrate ligands, these may represent a divergent class of polyunsaturated LCFAs, not derived from arachidonate, whose precise identification represents an exciting avenue for future research.

Eip75 mutations specifically affecting the isoform A *(E75A)* have a lethal profile similar to the one observed in oenocyte-less larvae (Bialecki, Shilton et al. 2002). Like oenocyte-less larvae, the majority of *E75A* larvae die during L2 or the L2/L3 moult, with only a few survivors reaching L3 and early pupal stages. Also like oenocyte-less larvae, some of the *E75A* larvae display a premature arrest in feeding and precocious wandering behaviour.

I attempted to rescue the oenocyte-less moulting phenotype by ecdysteroid treatment, in a similar way as Bialecki et al. did for *E75A* mutants. However, in contrast to their *Eip75B* study, oenocyte-less larvae were only partially rescued by this treatment. An early hypothesis suggested that oenocytes may be involved in the activation of ecdysone to 20E (Section 1.7.4). As previously stated in Section 1.6.3, more recent *in vivo* experiments propose that other peripheral tissues, such as the epidermis, midgut, Malpighian tubules and FB, are responsible for the activation of ecdysone into 20-E (Petryk, Warren et al. 2003). Thus, my ecdysteroid rescue experiments argue against the possibility that oenocytes are required for the activation, as both ecdysone or 20-E addition produce only a mild delay in lethality, rather than a full rescue.

An alternative hypothesis is that oenocytes could degrade rather than activate ecdysteroids (See Section 3.5). However, when fed ecdysteroids, a fraction of otherwise arrested oenocyteless-larvae were induced to begin the L2-L3 moult. If the degradation hypothesis were true, it is difficult (but not impossible) to understand how increasing the ecdysteroid titre in oenocyte-less larvae would partially rescue a moulting defect. *In vitro* salivary gland studies have shown that many late larval puffs appear prematurely when 20-E is removed (reviewed by (Riddiford 1993), suggesting that the fall of ecdysteroid levels is as important as their rise. Similarly, some mid-prepupal puffs of cultured salivary glands appear only in the absence of 20-E while others respond to 20-E only after this ecdysteroid free period (reviewed by (Riddiford 1993). Nonetheless, to clearly resolve this issues it would be useful to measure the ecdysterone and 20-E levels in oenocyte-less larvae (Kraminsky, Clark et al. 1980; Maroy, Koczka et al. 1980; Bialecki, Shilton et al. 2002). The use of lower

ecdysteroid concentrations and other feeding regimes in the hormone-rescue experiments may also provide extra information. In addition, a survey of the spatio-temporal pattern of ecdysteroid activation by means of an Ecdysone-Response Element reporter gene *(7xEcRE-lacZ)* would help to clarify this issue (Brennan, Ashburner et al. 1998; Munroe, Paine-Saunders et al. 1998; Ghbeish and McKeown 2002; Kozlova and Thummel 2003).

5.2.4 The *"pumpless"* Phenotype: A Possible Implications Link with Lipid Metabolism

In addition to the shared moulting defects between oenocyte-less larvae and *E75* mutants, oenocyte-less larvae display a second phenotype consisting on the suppression of food intake and wandering behaviour,. This phenotype has previously been associated with defective amino-acid catabolism (Zinke, Kirchner et al. 1999). Zinke and colleagues described the expression pattern and mutational analysis of a gene encoding a glycine cleavage system subunit (GCS), an enzyme involved in glycine catabolism by degradation into ammonia, carbon dioxide and one carbon unit (Mathews and van Holde 1990). GCS is specifically expressed in the FB during embryogenesis and larval stages with the highest level of expression at L2. Apparent GCS mutants were termed *pumpless (ppl)* as the first allele was identified as defective for the swallowing of food (Zinke, Kirchner et al. 1999). *ppl* mutants accumulate food in the pharynx but very little passes into the midgut despite the lack of a physical blockage in the foregut. This feeding defect is very similar to that of oenocyte-less larvae. It is not revealed before late L1 and it is accompanied by a variable decrease in body size. Also similar to oenocyte-less larvae, the majority of arrested *ppl* mutants die within 3 days, and display a premature wandering behaviour. In *ppl* larvae, the arrest in food intake is not accompanied by a normal physiological response to starvation, as seen by the lack of upregulation of two starvation marker genes *Lipase 3* and *phosphoenolpyruvate carboxykinase*. Zinke and collegues concluded that *ppl* may be implicated "in mediating the normal sequence of events, including those that are responsive to ecdysone, that lead to pupariation". However, in these published studies the identification of the ppl gene responsible for the phenotype was based primarily on the insertion mapping of 2 P-elements. The first of the insertions used, *ppl[06913]*, landed into *Ecdysone induce-protein 78 (Eip78)* and is accompanied by a deletion of half of the *Eip78* locus, *GCS* and *Acyl Coenzyme A Synthase (AcCoAS or*

AcS). The second P-element used, *ppl[00217],* was reported by Zinke et al. to be inserted 451 bp downstream of *GCS* and 32 bp upstream of one of the transcripts of *AcCoAS*.I have recently revisited the *ppl[00217]* insertion site data using release 3.0 of the genome. I find that *ppl[00217]* is inserted into the first exon of *AcCoAS* and not upstream of this gene as previously thought (Figure 5.1). Acyl-CoA synthases are enzymes involved in the first step of mitochondrial and peroxisomal ß-oxidations (Figure 1.2). These findings suggest that the *ppl* phenotype may not be caused by the lack of *GCS* function but rather by removing the activity of *AcCoAS*, and the consequent defects in lipid catabolism. Moreover, enzymes encoded by four oenocyte-genes involved in peroxisomal ß-oxidation: *COT, Pristanoyl-CoA oxidase, Catalase, CG11151* (highlighted in bold in Figure 1.2) are predicted to act downstream of an Acyl-CoA synthase. This raises the speculative possibility that the so-called *ppl* phenotype and that of oenocyte-less larvae result from defects at different points within the same lipid pathway. Future anaylsis of *AcCoAS* function and whether it is expressed in FB, oenocytes or other tissues clearly represents an important avenue of research.

5.2.5 Are Oenocytes or Fat Body the Insect Equivalent of Vertebrate Liver?

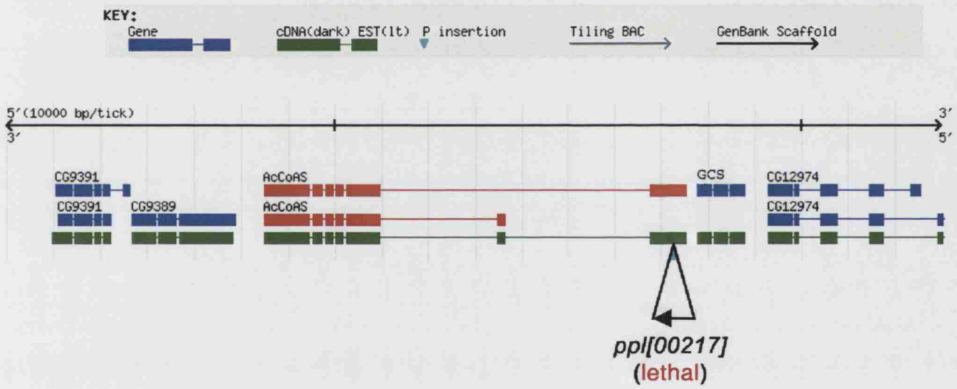
Previous studies have identified the FB as functionally a analogous tissue to both mammalian liver and adipocytes. In this regard, it is known that the FB accumulates lipids, in the form of intracellular droplets and also carbohydrates and proteins (Dean, Locke et al. 1985). The similarity between insect FB and mammalian adipocytes and hepatocytes is based on the expression of a handful of related genes. For example, the FB expresses *Lsd2*, a gene with a lean mutant phenotype that encodes a PAT domain-molecule (Gronke, Beller et al. 2003). Vertebrate PAT-containing molecules, such as Perilipin, are expressed in adipocytes where they are associated with intracellular lipid droplets. Other *Drosophila* gene productsexpressed in FB, whose orthologs are expressed in adipocytes include Alcohol dehydrogenase (Adh, reviewed in (Sondergaard 1993), amino-acid storage proteins, such as the female-specific yolk proteins (YPs, reviewed in (Karpen and Spradling 1992) and plasma proteins, such as the IGF-binding protein called Acid Labile Subunit (ALS, (Colombani, Raisin et al. 2003).

The results presented here suggest that insect oenocytes also fulfil some of the roles in lipid metabolism that in vertebrates are played by hepatocytes. In

Figure 5.1 The *pumpless(ppl)* Allele *ppl[00217]* Is Inserted into *Acetyl CoenzymeA Synthase (AcCoAS)* and Not *Glycine Cleavage System Subunit*

Although the *ppl* phenotype has previously attributed to GCS, *ppl[00217]* is a P-element insertion into first exon of one of the two isoforms of *AcCoAS.* The other *ppl* allele not shown in this diagram, *ppl[06913],* is a P-element insertion with an associated deletion of *GCS*, *AcCoAS* and part of *Eip78* locus.

A


KEY:
Gene
cDNA(dark) EST(lt) P insertion
Tiling BAC
GenBank Scaffold
5′(10000 bp/tick)
3′
3′
5′
CG9391
CG9391
CG9389
AcCoAS
AcCoAS
GCS
CG12974
CG12974
ppl[00217]
(lethal)

particular, the degradation of FAs by ß-oxidation appears to be an emportant oenocyte specialisation. Taking all the data discussed in the previous sections and by analogy to the vertebrate liver, a general and very speculative scheme of the roles of oenocyte genes in lipid metabolism can be constructed (Figure 5.2).

Whether the oenocytes are involved in other liver functions, such as glycogen storage, amino-acid storage, blood (hemolymph) detoxification or production of plasma proteins, remains to be tested. At present, the favoured hypothesis is that, in insects, liver functions are split between the FB and the oenocytes. An integrated physiological approach is now required to test how these two tissues communicate and coordinate the various steps in lipid metabolism.

5.3 Relating Lipid processing and Moulting with Previous Hypotheses for Oenocyte Function

The results from the gene identification screens, support the idea that oenocytes are involved in lipid modification and degradation. How might this new biochemical function for oenocytes be related to the classic hypotheses for the role of this cell-type?

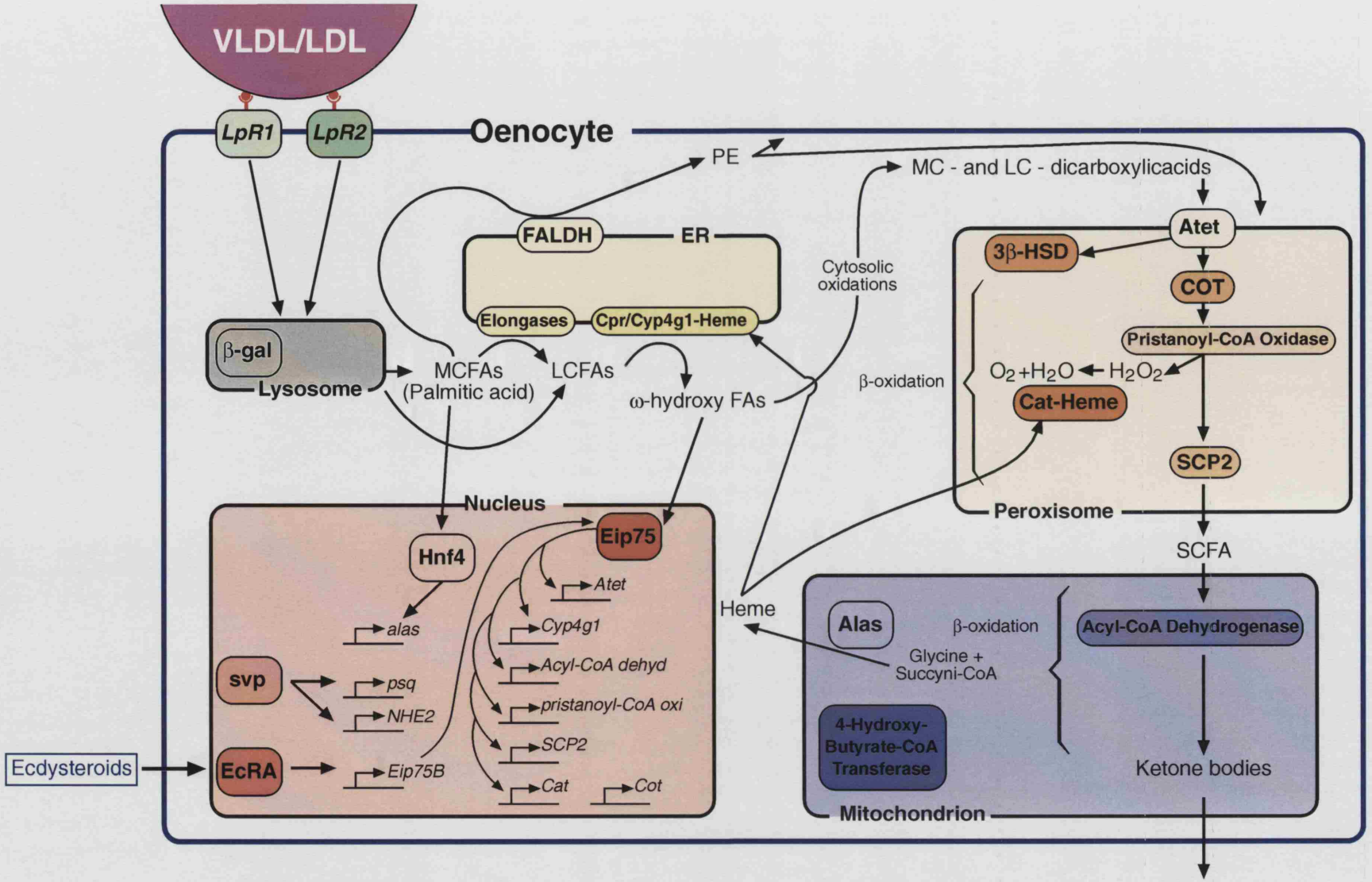
5.3.1 Possible Involvement of Oenocytes in Respiration

As first proposed by Glaser (1912), Oenocytes have been implicated in the respiratory process (Section 1.7.4). Consistent with this hypothesis, the molecular approach I have taken identified Catalase and several oxidases in oenocytes. However, research performed since Glaser's seminal work has shown that catalases and oxidases are involved in lipid degradation as well as respiration. The link between respiration and lipid processing extends to three other oenocyte genes, *Alas, Hnf4* and *svp*, which have been implicated in hypoxic responses (Galson, Tsuchiya et al. 1995; Guillemin and Krasnow 1997; Ruiz de Mena, Fernandez-Moreno et al. 1999) and, more recently *Cyp4g1* was identified as one of only two only genes to be upregulated in hypoxic larvae (Zhou, Lambert et al. 2003). Thus, although it has been difficult to disentangle the molecular machinery involved in hypoxia and lipid metabolism, the possibility that oenocytes play a role in hypoxia remains open.

Figure 5.2 Speculative Model for Oenocyte Function

Oenocytes may play a role in lipid catabolism analogous to that played by vertebrates hepatocytes. In this model, oenocytes capture lipoprotein structures (VLDL/LDL-like) from the hemolymph by means of two Lipophorin receptors (LpR1 and LpR2). Following lysosomal degradation, free medium-chain and long-chain fatty acids (MCFAs and LCFAs) are released into the cytoplasm, where they are modified by ER enzymes (FALDH, Elongases, Cpr/Cyp4g1). One of the products of such reactions, phosphatidylethanolamine (PE) gets incorporated into different cellular membranes. Some products of these ER reactions may act as intracrine ligands for Eip75 and possible other nuclear receptors, leading to the transcriptional activation of a battery of genes coding for enzymes involved in peroxisomal and mitochondrial ß-oxidation of FAs. Three other nuclear hormone receptors (Hnf4, svp and EcR-A) may regulate the expression of *Alas*, *psq*, *NHE2* and *Eip75B* itself. In particular, EcR-A may provide a link by which lipid metabolism could be coordinated with developmental progression and moulting.

All genes and gene products depicted here identified from the oenocyte screen in this work or were previously shown to be expressed in oenocytes. The regulatory relationships shown in this diagram has been assembled from multiple references. In some cases direct evidence exists for genetic relationships occurring in *Drosophila*. In many other instances, this is extrapolated from the vertebrate hepatocyte literature. Some arrows represent more than one biochemical reaction step.


VLDL/LDL
LpR1
LpR2
Oenocyte
PE
MC - and LC - dicarboxylicacids
FALDH
ER
Elongases
Cpr/Cyp4g1-Heme
Cytosolic oxidations
Atet
3β-HSD
COT
Pristanoyl-CoA Oxidase
β-gal
Lysosome
MCFAs (Palmitic acid)
LCFAs
ω-hydroxy FAs
β-oxidation
O2+H2O
H2O2
Cat-Heme
SCP2
Peroxisome
Nucleus
Hnf4
Eip75
Atet
alas
Cyp4g1
Acyl-CoA dehyd
svp
psq
NHE2
pristanoyl-CoA oxi
SCP2
EcRA
Eip75B
Cat
Cot
Ecdysteroids
Heme
SCFA
Alas
β-oxidation
Glycine + Succyni-CoA
Acyl-CoA Dehydrogenase
4-Hydroxy-Butyrate-CoA Transferase
Ketone bodies
Mitochondrion

5.3.2 Relationship between Oenocyte Function and Moulting

Previous studies have also implicated the oenocytes in two aspects of the moulting process and also in the regulation of hemolymph composition (Section 1.7.4). The results presented here are consistent with both hypotheses. Ablation of the oenocytes results in a moulting phenotype, while the genes they express suggest that oenocytes modify the hemolymph by capturing lipoprotein complexes and degrading their lipid components. Nevertheless, the question remains as to how these two findings might, if at all, be linked.

Wigglesworth proposed that oenocytes might to manufacture components of the cuticle (Section 1.7.4). The results of this work remain consistent with this hypothesis, as many of the products of the oenocyte lipid-processing enzymes might contribute to the lipid components of the cuticle. Moreover, some persistent L3 oenocyte-less larvae do appear to show a defective cuticle, that is very transparent. However, I can not rule out that this is the result of reduced opacity of internal organs, in particular the smaller-than-normal FB.

Thus, lipid degradation enzymes in oenocytes might be required for recycling cuticle lipid components prior to each moult, explaining the defective moulting seen in oenocyte-less larvae.

5.4 Concluding Remarks

Here, I have established an effective system for specifically eliminating the larval oenocytes. This involved considerable optimisation of the *GAL4/UAS-rpr* system. Data from the ablation experiments indicates that oenocytes play an essential role during larval life. Oenocyte-less larvae are not able to survive past L3 and develop asynchronously. They display a moulting phenotype that can not be rescued by ecdysteroid treatment and show reduced food intake and precocious wandering-like behaviours.

By enhancer trapping and an *in silico* screen of *in situ* patterns, 35 different oenocyte genes were identified, including seven that were highly oenocyte-specific. Strikingly, about half of these genes encode molecules involved either in the regulation of lipid metabolism or directly in lipid modification and degradation. These results indicate the existence of several enzymatic pathways of lipid metabolism that

are conserved between insects and vertebrates. Moreover, they strongly suggest that insect oenocytes are specialised for lipid modification and degradation and play a role analogous to that of the vertebrate hepatocytes.

The results presented here also suggest that regulated lipid processing is required for normal developmental progression. Future studies aimed at identifying the particular lipids synthesized by the oenocytes and characterising the individual functions of oenocyte-specific genes will clarify how these two processes are mechanistically linked.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abdelilah-Seyfried, S., Y. M. Chan, C. Zeng, N. J. Justice, S. Younger-Shepherd, L. E. Sharp, S. Barbel, S. A. Meadows, L. Y. Jan and Y. N. Jan (2000). "A gain-of-function screen for genes that affect the development of the Drosophila adult external sensory organ." Genetics **155**(2): 733-52.

Adamski, J., T. Normand, F. Leenders, D. Monte, A. Begue, D. Stehelin, P. W. Jungblut and Y. de Launoit (1995). "Molecular cloning of a novel widely expressed human 80 kDa 17 beta-hydroxysteroid dehydrogenase IV." Biochem J **311 (Pt 2)**: 437-43.

Akam, M. (1998). "Hox genes: from master genes to micromanagers." Curr Biol **8**(19): R676-8.

Alexandre, C., M. Lecourtois and J. Vincent (1999). "Wingless and Hedgehog pattern Drosophila denticle belts by regulating the production of short-range signals." Development **126**(24): 5689-98.

Antonenkov, V. D., P. P. Van Veldhoven, E. Waelkens and G. P. Mannaerts (1997). "Substrate specificities of 3-oxoacyl-CoA thiolase A and sterol carrier protein 2/3-oxoacyl-CoA thiolase purified from normal rat liver peroxisomes. Sterol carrier protein 2/3-oxoacyl-CoA thiolase is involved in the metabolism of 2-methyl-branched fatty acids and bile acid intermediates." J Biol Chem **272**(41): 26023-31.

Antoniewski, C., B. Mugat, F. Delbac and J. A. Lepesant (1996). "Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in Drosophila melanogaster." Mol Cell Biol **16**(6): 2977-86.

Aplin, A. C. and T. C. Kaufman (1997). "Homeotic transformation of legs to mouthparts by proboscipedia expression in Drosophila imaginal discs." Mech Dev **62**(1): 51-60.

Ashburner, M., C. Chihara, P. Meltzer and G. Richards (1974). Temporal control of puffing activity in polytene chromosomes. Cold Spring Harbour Symposia of Quantitative Biology.

Ashok, M., C. Turner and T. G. Wilson (1998). "Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators." Proc Natl Acad Sci U S A **95**(6): 2761-6.

Baker, K. D., L. M. Shewchuk, T. Kozlova, M. Makishima, A. Hassell, B. Wisely, J. A. Caravella, M. H. Lambert, J. L. Reinking, H. Krause, C. S. Thummel, T. M. Willson and D. J. Mangelsdorf (2003). "The Drosophila orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway." Cell **113**(6): 731-42.

Barak, Y., M. C. Nelson, E. S. Ong, Y. Z. Jones, P. Ruiz-Lozano, K. R. Chien, A. Koder and R. M. Evans (1999). "PPAR gamma is required for placental, cardiac, and adipose tissue development." Mol Cell **4**(4): 585-95.

Barrio, R., J. F. de Celis, S. Bolshakov and F. C. Kafatos (1999). "Identification of regulatory regions driving the expression of the Drosophila spalt complex at different developmental stages." Dev Biol **215**(1): 33-47.

Bassett, M. H., J. L. McCarthy, M. R. Waterman and T. J. Sliter (1997). "Sequence and developmental expression of Cyp18, a member of a new cytochrome P450 family from Drosophila." Mol Cell Endocrinol **131**(1): 39-49.

BDGP (2003). BDGP *in situ* homepage. **2003**.

BDGP Project Members (1994-1999). "Berkeley Drosophila Genome Project."

Begemann, G., A. M. Michon, L. vd Voorn, R. Wepf and M. Mlodzik (1995). "The Drosophila orphan nuclear receptor seven-up requires the Ras pathway for its function in photoreceptor determination." Development **121**(1): 225-35.

Belinski-Deutsch, S., D. Busson, C. Lamour-Audit, P. Porcheron, M. Moriniere and P. Berreur (1983). "Relations between ecdysteroid levels and pupal development in the ecd-1 temperature-sensitive mutant of *Drosophila melanogaster*." J. Insect Physiol. **29**: 509-514.

Berg, J., J. Tymoczko and L. Stryer (2001). Biochemistry. New York, Freeman and Co.

Bialecki, M., A. Shilton, C. Fichtenberg, W. A. Segraves and C. S. Thummel (2002). "Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in Drosophila." Dev Cell **3**(2): 209-20.

Bodenstein, D. (1950). The Postembryonic Development of Drosophila. Biology of Drosophila. M. Demerec. New York, John Wiley & Sons.

Brand, A. H., A. S. Manoukian and N. Perrimon (1994). "Ectopic expression in Drosophila." Methods Cell Biol **44**: 635-54.

Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of

altering cell fates and generating dominant phenotypes." Development **118**(2): 401-15.

Brennan, C. A., M. Ashburner and K. Moses (1998). "Ecdysone pathway is required for furrow progression in the developing Drosophila eye." Development **125**(14): 2653--2664.

Breyer, M. D. and R. M. Breyer (2000). "Prostaglandin E receptors and the kidney." Am J Physiol Renal Physiol **279**(1): F12-23.

Britton, J. S. and B. A. Edgar (1998). "Environmental control of the cell cycle in Drosophila: nutrition activates mitotic and endoreplicative cells by distinct mechanisms." Development **125**(11): 2149-58.

Brodu, V., P. R. Elstob and A. P. Gould (2002). "abdominal A specifies one cell type in Drosophila by regulating one principal target gene." Development **129**(12): 2957-63.

Bun-ya, M., Y. Muro, T. Niki, J. Kondo and T. Kamiryo (2000). "New aspects of sterol carrier protein 2 (nonspecific lipid-transfer protein) in fusion proteins and in peroxisomes." Cell Biochem Biophys **32**: 107-16.

Buszczak, M. and W. A. Segraves (1998). "Drosophila metamorphosis: the only way is USP?" Curr Biol **8**(24): R879-82.

Campos-Ortega, J. A. and V. Hartenstein (1997). The Embryonic Development of Drosophila Melanogaster. Berlin, Springer-Verlag.

Carey, J. R., P. Liedo, H. G. Muller, J. L. Wang and J. W. Vaupel (1998). "Dual modes of aging in Mediterranean fruit fly females." Science **281**(5379): 996-8.

Carton, Y., F. Frey, D. W. Stanley, E. Vass and A. J. Nappi (2002). "Dexamethasone inhibition of the cellular immune response of Drosophila melanogaster against a parasitoid." J Parasitol **88**(2): 405-7.

Chartier, A., S. Zaffran, M. Astier, M. Semeriva and D. Gratecos (2002). "Pericardin, a Drosophila type IV collagen-like protein is involved in the morphogenesis and maintenance of the heart epithelium during dorsal ectoderm closure." Development **129**(13): 3241-53.

Chavez, V. M., G. Marques, J. P. Delbecque, K. Kobayashi, M. Hollingsworth, J. Burr, J. E. Natzle and M. B. O'Connor (2000). "The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels." Development **127**(19):

4115-26.

Chawla, A., C. H. Lee, Y. Barak, W. He, J. Rosenfeld, D. Liao, J. Han, H. Kang and R. M. Evans (2003). "PPARdelta is a very low-density lipoprotein sensor in macrophages." Proc Natl Acad Sci U S A **100**(3): 1268-73.

Chawla, A., J. J. Repa, R. M. Evans and D. J. Mangelsdorf (2001). "Nuclear receptors and lipid physiology: opening the X-files." Science **294**(5548): 1866-70.

Cheon, H. M., S. J. Seo, J. Sun, T. W. Sappington and A. S. Raikhel (2001). "Molecular characterization of the VLDL receptor homolog mediating binding of lipophorin in oocyte of the mosquito Aedes aegypti." Insect Biochem Mol Biol **31**(8): 753-60.

Choi, S. J., D. H. Oh, C. S. Song, A. K. Roy and B. Chatterjee (1995). "Molecular cloning and sequence analysis of the rat liver carnitine octanoyltransferase cDNA, its natural gene and the gene promoter." Biochim Biophys Acta **1264**(2): 215-22.

Chyb, S., P. Raghu and R. C. Hardie (1999). "Polyunsaturated fatty acids activate the Drosophila light-sensitive channels TRP and TRPL." Nature **397**(6716): 255-9.

Colombani, J., S. Raisin, S. Pantalacci, T. Radimerski, J. Montagne and P. Leopold (2003). "A nutrient sensor mechanism controls Drosophila growth." Cell **114**(6): 739-49.

Crispi, S., E. Giordano, P. P. D'Avino and M. Furia (1998). "Cross-talking among Drosophila nuclear receptors at the promiscuous response element of the ng-1 and ng-2 intermolt genes." J Mol Biol **275**(4): 561-74.

Crispi, S., E. Giordano, P. P. D'Avino, I. Peluso and M. Furia (2001). "Functional analysis of regulatory elements controlling the expression of the ecdysone-regulated Drosophila ng-1 gene." Mech Dev **100**(1): 25-35.

Croes, K., M. Casteels, P. P. van Veldhoven and G. P. Mannaerts (1995). "Evidence for the importance of iron in the alpha-oxidation of 3-methyl-substituted fatty acids in the intact cell." Biochim Biophys Acta **1255**(1): 63-7.

Cui, X., H. Kawashima, T. B. Barclay, J. M. Peters, F. J. Gonzalez, E. T. Morgan and H. W. Strobel (2001). "Molecular cloning and regulation of expression of two novel mouse CYP4F genes: expression in peroxisome proliferator-activated receptor alpha-deficient mice upon lipopolysaccharide and clofibrate

challenges." J Pharmacol Exp Ther **296**(2): 542-50.

Culi, J. and R. S. Mann (2003). "Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in Drosophila." Cell **112**(3): 343-54.

Dai, J. D. and L. I. Gilbert (1991). "Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of Drosophila melanogaster: a cytophysiological analysis of the ring gland." Dev Biol **144**(2): 309-26.

Dantuma, N. P., M. Potters, M. P. De Winther, C. P. Tensen, F. P. Kooiman, J. Bogerd and D. J. Van der Horst (1999). "An insect homolog of the vertebrate very low density lipoprotein receptor mediates endocytosis of lipophorins." J Lipid Res **40**(5): 973-8.

Davis, R. L. (2001). "Mushroom bodies, Ca(2+) oscillations, and the memory gene amnesiac." Neuron **30**(3): 653-6.

Dean, M., Y. Hamon and G. Chimini (2001). "The human ATP-binding cassette (ABC) transporter superfamily." J Lipid Res **42**(7): 1007-17.

Dean, M., A. Rzhetsky and R. Allikmets (2001). "The human ATP-binding cassette (ABC) transporter superfamily." Genome Res **11**(7): 1156-66.

Dean, R., M. Locke and J. Collins (1985). Structure of the fat body. Comprehensive Insect Physiology, Biochemistry and Pharmacology. G. Kerkut and L. I. Gilbert. New York, Pergamonn Press: 155-210.

DeZazzo, J., S. Xia, J. Christensen, K. Velinzon and T. Tully (1999). "Developmental expression of an amn(+) transgene rescues the mutant memory defect of amnesiac adults." J Neurosci **19**(20): 8740-6.

Dobrosotskaya, I. Y., A. C. Seegmiller, M. S. Brown, J. L. Goldstein and R. B. Rawson (2002). "Regulation of SREBP processing and membrane lipid production by phospholipids in Drosophila." Science **296**(5569): 879-83.

Dorn, A. and F. Romer (1976). "Structure and function of prothoracic glands and oenocytes in embryos and last larval instars of Oncopeltus fasciatus Dallas (Insecta, Heteroptera)." Cell Tissue Res **171**(3): 331-50.

Dreyer, C., H. Keller, A. Mahfoudi, V. Laudet, G. Krey and W. Wahli (1993). "Positive regulation of the peroxisomal beta-oxidation pathway by fatty acids through activation of peroxisome proliferator-activated receptors (PPAR)."

Biol Cell 77(1): 67-76.

Dunkov, B. C., R. Rodriguez-Arnaiz, B. Pittendrigh, R. H. ffrench-Constant and R. Feyereisen (1996). "Cytochrome P450 gene clusters in Drosophila melanogaster." Mol Gen Genet **251**(3): 290-7.

Eastham, L. (1929). "The Post-Embryonic Development of Phaenoserphus viator Hal. (Proctotrypoidea), a Parasite of the Larva of Pterostichus niger (Carabidae), with Notes on the Anatomy of the Larva." Parasitology **21**(1 & 2): 1-21.

Elstob, P. R. (2001). *Hox* Gene Function and Cell Identity in *Drosophila.* Ph.D. Thesis. National Institute for Medical Research. London, University of London.

Elstob, P. R., V. Brodu and A. P. Gould (2001). "*spalt*-dependent switching between two cell fates that are induced by the *Drosophila* EGF receptor." Development **128**: 723-732.

Emanuelsson, O., A. Elofsson, G. von Heijne and S. Cristobal (2003). "In silico prediction of the peroxisomal proteome in fungi, plants and animals." J Mol Biol **330**(2): 443-56.

Everett, L., A. Galli and D. Crabb (2000). "The role of hepatic peroxisome proliferator-activated receptors (PPARs) in health and disease." Liver **20**(3): 191-9.

Feany, M. B. and W. G. Quinn (1995). "A neuropeptide gene defined by the Drosophila memory mutant amnesiac." Science **268**(5212): 869-73.

Fernandez-Rachubinski, F. and L. Fliegel (2001). "COUP-TFI and COUP-TFII regulate expression of the NHE through a nuclear hormone responsive element with enhancer activity." Eur J Biochem **268**(3): 620-34.

Fontaine, C., G. Dubois, Y. Duguay, T. Helledie, N. Vu-Dac, P. Gervois, F. Soncin, S. Mandrup, J. C. Fruchart, J. Fruchart-Najib and B. Staels (2003). "The orphan nuclear receptor Rev-erbalpha is a PPARgamma target gene and promotes PPARgamma -induced adipocyte differentiation." J Biol Chem.

Forman, B. M., J. Chen and R. M. Evans (1997). "Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta." Proc Natl Acad Sci U S A **94**(9): 4312-7.

Forman, B. M., P. Tontonoz, J. Chen, R. P. Brun, B. M. Spiegelman and R. M. Evans

(1995). "15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma." Cell **83**(5): 803-12.

Fourcade, S., S. Savary, S. Albet, D. Gauthe, C. Gondcaille, T. Pineau, J. Bellenger, M. Bentejac, A. Holzinger, J. Berger and M. Bugaut (2001). "Fibrate induction of the adrenoleukodystrophy-related gene (ABCD2): promoter analysis and role of the peroxisome proliferator-activated receptor PPARalpha." Eur J Biochem **268**(12): 3490-500.

Freeman, M. (1994). "The spitz gene is required for photoreceptor determination in the Drosophila eye where it interacts with the EGF receptor." Mech Dev **48**(1): 25-33.

Freeman, M. R., A. Dobritsa, P. Gaines, W. A. Segraves and J. R. Carlson (1999). "The dare gene: steroid hormone production, olfactory behavior, and neural degeneration in Drosophila." Development **126**(20): 4591-602.

Fu, J., S. Gaetani, F. Oveisi, J. Lo Verme, A. Serrano, F. Rodriguez De Fonseca, A. Rosengarth, H. Luecke, B. Di Giacomo, G. Tarzia and D. Piomelli (2003). "Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha." Nature **425**(6953): 90-3.

Fujiki, Y. (1997). "Molecular defects in genetic diseases of peroxisomes." Biochim Biophys Acta **1361**(3): 235-50.

Funk, C. D. (2001). "Prostaglandins and leukotrienes: advances in eicosanoid biology." Science **294**(5548): 1871-5.

Gade, G., K. H. Hoffmann and J. H. Spring (1997). "Hormonal regulation in insects: facts, gaps, and future directions." Physiol Rev **77**(4): 963-1032.

Gallant, P., Y. Shiio, P. F. Cheng, S. M. Parkhurst and R. N. Eisenman (1996). "Myc and Max homologs in Drosophila." Science **274**(5292): 1523-7.

Galson, D. L., T. Tsuchiya, D. S. Tendler, L. E. Huang, Y. Ren, T. Ogura and H. F. Bunn (1995). "The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1." Mol Cell Biol **15**(4): 2135-44.

Gandhi, R., E. Varak and M. L. Goldberg (1992). "Molecular analysis of a cytochrome P450 gene of family 4 on the Drosophila X chromosome." DNA Cell Biol **11**(5): 397-404.

Gausz, J., H. Gyurkovics, G. Bencze, A. A. Awad, J. J. Holden and D. Ish-Horowicz (1981). "Genetic characterization of the region between 86F1,2 and 87B15 on chromosome 3 of Drosophila melanogaster." Genetics **98**(4): 775-89.

Gauthier, A., G. Vassiliou, F. Benoist and R. McPherson (2003). "Adipocyte low density lipoprotein receptor-related protein gene expression and function is regulated by peroxisome proliferator-activated receptor gamma." J Biol Chem **278**(14): 11945-53.

Gerhardt, A., I. Cinkaya, D. Linder, G. Huisman and W. Buckel (2000). "Fermentation of 4-aminobutyrate by Clostridium aminobutyricum: cloning of two genes involved in the formation and dehydration of 4-hydroxybutyryl-CoA." Arch Microbiol **174**(3): 189-99.

Ghbeish, N. and M. McKeown (2002). "Analyzing the repressive function of ultraspiracle, the Drosophila RXR, in Drosophila eye development." Mech. Dev. **111**(1-2): 89--98.

Giannakou, M. E. and J. A. Dow (2001). "Characterization of the Drosophila melanogaster alkali-metal/proton exchanger (NHE) gene family." J Exp Biol **204**(Pt 21): 3703-16.

Gilbert, L. I., Q. Song and R. Rybczynski (1997). "Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity." Invert Neurosci **3**(2-3): 205-16.

Ginsberg, H. N. (2002). "New perspectives on atherogenesis: role of abnormal triglyceride-rich lipoprotein metabolism." Circulation **106**(16): 2137-42.

Girnun, G. D., F. E. Domann, S. A. Moore and M. E. Robbins (2002). "Identification of a functional peroxisome proliferator-activated receptor response element in the rat catalase promoter." Mol Endocrinol **16**(12): 2793-801.

Glaser, R. (1912). "A Contribution to our Knowledge of the Function of the Oenocytes of Insects." Biol. Bull. **23**(3): 213-224.

Gooding, H., S. Choksi and M. Bender (2000). Identification of mutants in the Drosophila prohormone convertase gene amontillado (amon). A. Dros. Res. Conf.

Goodman, W. G. (1990). Biosynthesis, titer regulation, and transport of juvenile hormones. Morphogenetic Hormones of Arthropods: Discoveries, Syntheses, Metabolism, Evolution, Modes of Action and Techniques. A. P. Gupta. New

Brunswick, Rutgers University Press.

Gorski, S. M., S. Chittaranjan, E. D. Pleasance, J. D. Freeman, C. L. Anderson, R. J. Varhol, S. M. Coughlin, S. D. Zuyderduyn, S. J. M. Jones and M. A. Marra (2003). "A SAGE approach to discovery of genes involved in autophagic cell death." Curr. Biol. **13**(4): 358--363.

Gould, A. (2000). "Flybase FBrf0126870."

Gould, A. P., P. R. Elstob and V. Brodu (2001). "Insect oenocytes: a model system for studying cell-fate specification by Hox genes." J Anat **199**(Pt 1-2): 25-33.

Graber, V. (1873). "Ueber den propulsatorischen Apparat der Insecten." Archiv f. mikrosk. Anatomie **9**: 129-196.

Greenspan, R. J. (1997). Fly Pushing. The Theory and Practice of *Drosophila* Genetics. New York, Cold Spring Harbor Laboratory Press.

Grieneisen, M. L. (1994). "Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans." Insect Biochem Mol Biol **24**: 115-132.

Griswold, C. M., A. L. Matthews, K. E. Bewley and J. W. Mahaffey (1993). "Molecular characterization and rescue of acatalasemic mutants of Drosophila melanogaster." Genetics **134**(3): 781-8.

Gronke, S., M. Beller, S. Fellert, H. Ramakrishnan, H. Jackle and R. P. Kuhnlein (2003). "Control of Fat Storage by a Drosophila PAT Domain Protein." Curr Biol **13**(7): 603-6.

Guillemin, K. and M. A. Krasnow (1997). "The hypoxic response: huffing and HIFing." Cell **89**(1): 9-12.

Hagedorn, H. H. (1989). Physiological roles of hemolymph ecdysteroids in the adult insect. Ecdysone - from Chemistry to Mode of Action. J. Koolman. Stuttgart, Georg Thieme Verlag: 279-289.

Hall, B. L. and C. S. Thummel (1998). "The RXR homolog ultraspiracle is an essential component of the Drosophila ecdysone receptor." Development **125**(23): 4709-17.

Hartfelder, K. (2000). "Insect juvenile hormone: from "status quo" to high society." Braz J Med Biol Res **33**(2): 157-77.

Hashimoto, T. (2000). "Peroxisomal beta-oxidation enzymes." Cell Biochem Biophys **32 Spring**: 63-72.

Hayashi, S., K. Ito, Y. Sado, M. Taniguchi, A. Akimoto, H. Takeuchi, T. Aigaki, F. Matsuzaki, H. Nakagoshi, T. Tanimura, R. Ueda, T. Uemura, M. Yoshihara and S. Goto (2002). "GETDB, a database compiling expression patterns and molecular locations of a collection of Gal4 enhancer traps." Genesis **34**(1-2): 58-61.

Hettema, E. H., C. W. van Roermund, B. Distel, M. van den Berg, C. Vilela, C. Rodrigues-Pousada, R. J. Wanders and H. F. Tabak (1996). "The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of Saccharomyces cerevisiae." Embo J **15**(15): 3813-22.

Hewes, R. S., A. M. Schaefer and P. H. Taghert (2000). "The cryptocephal gene (ATF4) encodes multiple basic-leucine zipper proteins controlling molting and metamorphosis in Drosophila." Genetics **155**(4): 1711-23.

Hidalgo, A., J. Urban and A. H. Brand (1995). "Targeted ablation of glia disrupts axon tract formation in the Drosophila CNS." Development **121**(11): 3703-12.

Hintermann, E., N. C. Grieder, R. Amherd, D. Brodbeck and U. A. Meyer (1996). "Cloning of an arylalkylamine N-acetyltransferase (aaNAT1) from Drosophila melanogaster expressed in the nervous system and the gut." Proc Natl Acad Sci U S A **93**(22): 12315-20.

Hollande, C. (1914). "Les Cérodécytes ou "Oenocytes" des Insectes." Arch. D'Anat. Microsc. **16**: 1-66.

Hoshizaki, D. K., T. Blackburn, C. Price, M. Ghosh, K. Miles, M. Ragucci and R. Sweis (1994). "Embryonic fat-cell lineage in Drosophila melanogaster." Development **120**(9): 2489-99.

Hovemann, B. T., F. Sehlmeyer and J. Malz (1997). "Drosophila melanogaster NADPH-cytochrome P450 oxidoreductase: pronounced expression in antennae may be related to odorant clearance." Gene **189**(2): 213-9.

Hua, Y. J., D. Bylemans, A. De Loof and J. Koolman (1994). "Inhibition of ecdysone biosynthesis in flies by a hexapeptide isolated from vitellogenic ovaries." Mol Cell Endocrinol **104**(1): R1-4.

Ikeya, T., M. Galic, P. Belawat, K. Nairz and E. Hafen (2002). "Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila." Curr Biol **12**(15): 1293-300.

Ip, Y. T., M. Levine and E. Bier (1994). "Neurogenic expression of snail is controlled by separable CNS and PNS promoter elements." Development **120**(1): 199-207.

Issemann, I. and S. Green (1990). "Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators." Nature **347**(6294): 645-50.

Jacobs, E. R. and D. C. Zeldin (2001). "The lung HETEs (and EETs) up." Am J Physiol Heart Circ Physiol **280**(1): H1-H10.

Janet, C. (1909). Sur L'Ontogenese de L'Insecte. Limoges, Ducourtieux et Gout.

Janowski, B. A., P. J. Willy, T. R. Devi, J. R. Falck and D. J. Mangelsdorf (1996). "An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha." Nature **383**(6602): 728-31.

Jarecki, J., E. Johnson and M. A. Krasnow (1999). "Oxygen regulation of airway branching in Drosophila is mediated by branchless FGF." Cell **99**(2): 211-20.

Jiang, N., A. S. Kolhekar, P. S. Jacobs, R. E. Mains, B. A. Eipper and P. H. Taghert (2000). "PHM is required for normal developmental transitions and for biosynthesis of secretory peptides in Drosophila." Dev Biol **226**(1): 118-36.

Johnson, E. A. and M. A. Krasnow (2001). Characterization of the HIF-1 hypoxia response pathway in vivo using genetic and genomic approaches. A. Dros. Res. Conf. 42.

Johnston, L. A., D. A. Prober, B. A. Edgar, R. N. Eisenman and P. Gallant (1999). "Drosophila myc regulates cellular growth during development." Cell **98**(6): 779-90.

Jones, G. (1995). "Molecular mechanism of action of juvenile hormone." Annual Review of Entomology **40**: 147-169.

Jones, N. A., Y. M. Kuo, Y. H. Sun and S. K. Beckendorf (1998). "The Drosophila Pax gene eye gone is required for embryonic salivary duct development." Development **125**(21): 4163-74.

Kaikaus, R. M., W. K. Chan, N. Lysenko, R. Ray, P. R. Ortiz de Montellano and N. M. Bass (1993). "Induction of peroxisomal fatty acid beta-oxidation and liver fatty acid-binding protein by peroxisome proliferators. Mediation via the cytochrome P-450IVA1 omega-hydroxylase pathway." J Biol Chem **268**(13): 9593-603.

Kal, A. J., E. H. Hettema, M. van den Berg, M. G. Koerkamp, L. van Ijlst, B. Distel

and H. F. Tabak (2000). "In silicio search for genes encoding peroxisomal proteins in Saccharomyces cerevisiae." Cell Biochem Biophys **32 Spring**: 1-8.

Karlson, P. (1963). "New concepts on the mode of action of hormones." Perspect Biol Med **6**(2): 203-14.

Karpen, G. H. and A. C. Spradling (1992). "Analysis of subtelomeric heterochromatin in the Drosophila minichromosome Dp1187 by single P element insertional mutagenesis." Genetics **132**(3): 737-53.

Kelson, T. L., J. R. Secor McVoy and W. B. Rizzo (1997). "Human liver fatty aldehyde dehydrogenase: microsomal localization, purification, and biochemical characterization." Biochim Biophys Acta **1335**(1-2): 99-110.

Kerber, B., S. Fellert and M. Hoch (1998). "Seven-up, the Drosophila homolog of the COUP-TF orphan receptors, controls cell proliferation in the insect kidney." Genes Dev **12**(12): 1781-6.

Kersten, S., J. Seydoux, J. M. Peters, F. J. Gonzalez, B. Desvergne and W. Wahli (1999). "Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting." J Clin Invest **103**(11): 1489-98.

Kingan, T. G., W. Gray, D. Zitnan and M. E. Adams (1997). "Regulation of ecdysis-triggering hormone release by eclosion hormone." J Exp Biol **200 (Pt 24)**: 3245-56.

Kliewer, S. A., J. M. Lehmann and T. M. Willson (1999). "Orphan nuclear receptors: shifting endocrinology into reverse." Science **284**(5415): 757-60.

Kliewer, S. A., J. M. Lenhard, T. M. Willson, I. Patel, D. C. Morris and J. M. Lehmann (1995). "A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation." Cell **83**(5): 813-9.

Kliewer, S. A., S. S. Sundseth, S. A. Jones, P. J. Brown, G. B. Wisely, C. S. Koble, P. Devchand, W. Wahli, T. M. Willson, J. M. Lenhard and J. M. Lehmann (1997). "Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma." Proc Natl Acad Sci U S A **94**(9): 4318-23.

Kniazeva, M., M. Sieber, S. McCauley, K. Zhang, J. L. Watts and M. Han (2003). "Suppression of the ELO-2 FA Elongation Activity Results in Alterations of the Fatty Acid Composition and Multiple Physiological Defects, Including

Abnormal Ultradian Rhythms, in Caenorhabditis elegans." Genetics **163**(1): 159-69.

Koller, G. (1928). "Die innere Sekretion bei wirbellosen Tieren." Biol. Rev. **4**: 269-306.

Koolman, J. (1990). "Ecdysteroids." Zoological Science **7**(563-580).

Koschevnikov, G. (1900). "Ueber den FettKoerper und die Oenocyten der Honigbiene (*Apis mellifera)*." Z. Anz., Bd. **23**(618): 657-661.

Kosman, D., Y. T. Ip, M. Levine and K. Arora (1991). "Establishment of the mesoderm-neuroectoderm boundary in the Drosophila embryo." Science **254**(5028): 118-22.

Kozlova, T. and C. S. Thummel (2000). "Steroid regulation of postembryonic development and reproduction in Drosophila." Trends Endocrinol Metab **11**(7): 276-80.

Kozlova, T. and C. S. Thummel (2003). "Essential roles for ecdysone signaling during Drosophila mid-embryonic development." Science **301**(5641): 1911-4.

Kraminsky, G. P., W. C. Clark, M. A. Estelle, R. D. Gietz, B. A. Sage, J. D. O'Connor and R. B. Hodgetts (1980). "Induction of translatable mRNA for dopa decarboxylase in Drosophila: an early response to ecdysterone." Proc Natl Acad Sci U S A **77**(7): 4175-9.

Kraut, R., K. Menon and K. Zinn (2001). "A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in Drosophila." Curr Biol **11**(6): 417-30.

Krey, G., O. Braissant, F. L'Horset, E. Kalkhoven, M. Perroud, M. G. Parker and W. Wahli (1997). "Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay." Mol Endocrinol **11**(6): 779-91.

Kubota, N., Y. Terauchi, H. Miki, H. Tamemoto, T. Yamauchi, K. Komeda, S. Satoh, R. Nakano, C. Ishii, T. Sugiyama, K. Eto, Y. Tsubamoto, A. Okuno, K. Murakami, H. Sekihara, G. Hasegawa, M. Naito, Y. Toyoshima, S. Tanaka, K. Shiota, T. Kitamura, T. Fujita, O. Ezaki, S. Aizawa, T. Kadowaki and et al. (1999). "PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance." Mol Cell **4**(4): 597-609.

Kuhnlein, R. P., G. Frommer, M. Friedrich, M. Gonzalez-Gaitan, A. Weber, J. F.

Wagner-Bernholz, W. J. Gehring, H. Jackle and R. Schuh (1994). "spalt encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the Drosophila embryo." Embo J **13**(1): 168-79.

Kuwana, H., K. Shimizu-Nishikawa, H. Iwahana and D. Yamamoto (1996). "Molecular cloning and characterization of the ABC transporter expressed in Trachea (ATET) gene from Drosophila melanogaster." Biochim Biophys Acta **1309**(1-2): 47-52.

Labbe-Bois, R. and P. Labbe (1990). Tetrapyrrole and heme biosynthesis in the yeast Saccharomyces cerevisiae. Biosynthesis of Heme and Chlorophylls. H. A. Dailey. New York, McGraw-Hill Publishing Co.: 235-285.

Lafont, R. (2000). "Understanding insect endocrine systems: molecular approaches." Entomologia Experimentalis et Applicata **97**: 123-136.

Landois, L. (1865). "Ueber die Fuction des Fettkörpers." Zeitschr. f. Wissensch. Zoologie **15**: 371-372.

Lazar, M. A., R. A. Hodin, D. S. Darling and W. W. Chin (1989). "A novel member of the thyroid/steroid hormone receptor family is encoded by the opposite strand of the rat c-erbA alpha transcriptional unit." Mol Cell Biol **9**(3): 1128-36.

Lazarow, P. B. (2003). "Peroxisome biogenesis: advances and conundrums." Curr Opin Cell Biol **15**(4): 489-97.

Lazarow, P. B. and C. De Duve (1976). "A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug." Proc Natl Acad Sci U S A **73**(6): 2043-6.

Lee, C. H., P. Olson and R. M. Evans (2003). "Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors." Endocrinology **144**(6): 2201-7.

Lee, C. Y., E. A. Clough, P. Yellon, T. M. Teslovich, D. A. Stephan and E. H. Baehrecke (2003). "Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila." Curr. Biol. **13**(4): 350--357.

Lee, Y. S. and R. W. Carthew (2003). "Making a better RNAi vector for Drosophila: use of intron spacers." Methods **30**(4): 322-9.

Lehmann, J. M., S. A. Kliewer, L. B. Moore, T. A. Smith-Oliver, B. B. Oliver, J. L.

Su, S. S. Sundseth, D. A. Winegar, D. E. Blanchard, T. A. Spencer and T. M. Willson (1997). "Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway." J Biol Chem **272**(6): 3137-40.

Leone, T. C., C. J. Weinheimer and D. P. Kelly (1999). "A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders." Proc Natl Acad Sci U S A **96**(13): 7473-8.

Lezzi, M. (1996). Chromosome puffing: Supramolecular aspects of ecdysone action. Metamorphosis: Post-Embryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. L. I. Gilbert, J. Tata and B. Atkinson. San Diego, Academic Press, Inc.

Li, J., G. Ning and S. A. Duncan (2000). "Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha." Genes Dev **14**(4): 464-74.

Li, T. and M. Bender (2000). "A conditional rescue system reveals essential functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in Drosophila." Development **127**(13): 2897-905.

Liang, Z. and M. D. Biggin (1998). "Eve and ftz regulate a wide array of genes in blastoderm embryos: the selector homeoproteins directly or indirectly regulate most genes in Drosophila." Development **125**(22): 4471-82.

Lim, H. and S. K. Dey (2002). "A novel pathway of prostacyclin signaling-hanging out with nuclear receptors." Endocrinology **143**(9): 3207-10.

Locke, M. (1969). "The ultrastructure of the oenocytes in the moult/intermoult cycle of an insect." Tissue and Cell **1**: 103-154.

Lohmann, I., N. McGinnis, M. Bodmer and W. McGinnis (2002). "The Drosophila Hox gene deformed sculpts head morphology via direct regulation of the apoptosis activator reaper." Cell **110**(4): 457-66.

Lohmann, I. and W. McGinnis (2002). "Hox Genes: it's all a matter of context." Curr Biol **12**(15): R514-6.

Londos, C., D. L. Brasaemle, C. J. Schultz, J. P. Segrest and A. R. Kimmel (1999). "Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells." Semin Cell Dev Biol **10**(1): 51-8.

Lopez, D., R. B. Irby and M. P. McLean (2003). "Peroxisome proliferator-activated receptor alpha induces rat sterol carrier protein x promoter activity through

two peroxisome proliferator-response elements." Mol Cell Endocrinol **205**(1-2): 169-84.

Maglich, J. M., A. Sluder, X. Guan, Y. Shi, D. D. McKee, K. Carrick, K. Kamdar, T. M. Willson and J. T. Moore (2001). "Comparison of complete nuclear receptor sets from the human, Caenorhabditis elegans and Drosophila genomes." Genome Biol **2**(8): RESEARCH0029.

Maibeche-Coisne, M., L. Monti-Dedieu, S. Aragon and C. Dauphin-Villemant (2000). "A new cytochrome P450 from Drosophila melanogaster, CYP4G15, expressed in the nervous system." Biochem Biophys Res Commun **273**(3): 1132-7.

Mannaerts, G. P., P. P. Van Veldhoven and M. Casteels (2000). "Peroxisomal lipid degradation via beta- and alpha-oxidation in mammals." Cell Biochem Biophys **32 Spring**: 73-87.

Manning, G. and M. A. Krasnow (1993). Development of the *Drosophila* tracheal system. The Develoment of *Drosophila melanogaster*. M. Bate and A. Martinez Arias. New York, Cold Spring Harbor Laboratory Press: 609-684.

Maroy, P., K. Koczka, E. Fekete and J. Vargha (1980). "Molting hormone titer of D. melanogaster larvae." D. I. S. **55**: 98--99.

Mathews, C. and K. van Holde (1990). Biochemistry. Redwood City, The Benjamin/Cummings Publications.

Matsubara, S., S. Yamamoto, K. Sogawa, N. Yokotani, Y. Fujii-Kuriyama, M. Haniu, J. E. Shively, O. Gotoh, E. Kusunose and M. Kusunose (1987). "cDNA cloning and inducible expression during pregnancy of the mRNA for rabbit pulmonary prostaglandin omega-hydroxylase (cytochrome P-450p-2)." J Biol Chem **262**(27): 13366-71.

McCallum, C. M., L. Comai, E. A. Greene and S. Henikoff (2000). "Targeting induced local lesions in genomes (TILLING) for plant functional genomics." Plant Physiol **123**(2): 439-42.

McGinnis, W. and R. Krumlauf (1992). "Homeobox genes and axial patterning." Cell **68**: 283-302.

Mesce, K. A. and S. E. Fahrbach (2002). "Integration of endocrine signals that regulate insect ecdysis." Front Neuroendocrinol **23**(2): 179-99.

Moore, M. S., J. DeZazzo, A. Y. Luk, T. Tully, C. M. Singh and U. Heberlein (1998).

"Ethanol intoxication in Drosophila: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway." Cell **93**(6): 997-1007.

Munroe, S., S. Paine-Saunders, S. Stowers and A. Rascle (1998). Flybase FBrf0102541.

Nagy, L., P. Tontonoz, J. G. Alvarez, H. Chen and R. M. Evans (1998). "Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma." Cell **93**(2): 229-40.

Narumiya, S., Y. Sugimoto and F. Ushikubi (1999). "Prostanoid receptors: structures, properties, and functions." Physiol Rev **79**(4): 1193-226.

Nebert, D. W. and D. W. Russell (2002). "Clinical importance of the cytochromes P450." Lancet **360**(9340): 1155-62.

Nijhout, H. F. (1994). Insect Hormones, Princeton University Press.

Nijhout, H. F. (2003). "The control of body size in insects." Dev Biol **261**(1): 1-9.

Nohturfft, A. and R. Losick (2002). "Cell biology. Fats, flies, and palmitate." Science **296**(5569): 857-8.

Nosjean, O. and J. A. Boutin (2002). "Natural ligands of PPARgamma: are prostaglandin J(2) derivatives really playing the part?" Cell Signal **14**(7): 573-83.

O'Neill, E. M., I. Rebay, R. Tjian and G. M. Rubin (1994). "The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway." Cell **78**(1): 137-47.

Okabe, M. and H. Okano (1997). "Two-step induction of chordotonal organ precursors in Drosophila embryogenesis." Development **124**(5): 1045-53.

Okita, R. T. and J. R. Okita (2001). "Cytochrome P450 4A fatty acid omega hydroxylases." Curr Drug Metab **2**(3): 265-81.

Oldham, S., R. Bohni, H. Stocker, W. Brogiolo and E. Hafen (2000). "Genetic control of size in Drosophila." Philos Trans R Soc Lond B Biol Sci **355**(1399): 945-52.

Orr, W. C., E. C. Orr, S. K. Legan and R. S. Sohal (1996). "Molecular analysis of the Drosophila catalase gene." Arch Biochem Biophys **330**(2): 251-8.

Orr, W. C. and R. S. Sohal (1992). "The effects of catalase gene overexpression on life span and resistance to oxidative stress in transgenic Drosophila melanogaster." Arch Biochem Biophys **297**(1): 35-41.

Pages, M., J. Rosello, J. Casas, E. Gelpi, N. Gualde and M. Rigaud (1986). "Cyclooxygenase and lipoxygenase-like activity in Drosophila melanogaster." Prostaglandins **32**(5): 729-40.

Park, Y., V. Filippov, S. S. Gill and M. E. Adams (2002). "Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency." Development **129**(2): 493-503.

Parviz, F., C. Matullo, W. D. Garrison, L. Savatski, J. W. Adamson, G. Ning, K. H. Kaestner, J. M. Rossi, K. S. Zaret and S. A. Duncan (2003). "Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis." Nat Genet **34**(3): 292-6.

Peet, D. J., S. D. Turley, W. Ma, B. A. Janowski, J. M. Lobaccaro, R. E. Hammer and D. J. Mangelsdorf (1998). "Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha." Cell **93**(5): 693-704.

Pelletier, G., S. Li, V. Luu-The, Y. Tremblay, A. Belanger and F. Labrie (2001). "Immunoelectron microscopic localization of three key steroidogenic enzymes (cytochrome P450(scc), 3 beta-hydroxysteroid dehydrogenase and cytochrome P450(c17)) in rat adrenal cortex and gonads." J Endocrinol **171**(2): 373-83.

Pena-Rangel, M. T., I. Rodriguez and J. R. Riesgo-Escovar (2002). "A misexpression study examining dorsal thorax formation in Drosophila melanogaster." Genetics **160**(3): 1035-50.

Peng, L., J. Arensburg, J. Orly and A. H. Payne (2002). "The murine 3beta-hydroxysteroid dehydrogenase (3beta-HSD) gene family: a postulated role for 3beta-HSD VI during early pregnancy." Mol Cell Endocrinol **187**(1-2): 213-21.

Perez, C. (1901). "Sur les oenocytes de la Fourmi rousse." Bulletin de la Societe Entomologique de France **19**: 351-353.

Perrimon, N., L. Engstrom and A. P. Mahowald (1985). "Developmental genetics of the 2C-D region of the Drosophila X chromosome." Genetics **111**(1): 23-41.

Peters, J. M., N. Hennuyer, B. Staels, J. C. Fruchart, C. Fievet, F. J. Gonzalez and J. Auwerx (1997). "Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor alpha-deficient mice." J Biol Chem **272**(43): 27307-12.

Petryk, A., J. T. Warren, G. Marques, M. P. Jarcho, L. I. Gilbert, J. Kahler, J. P. Parvy, Y. Li, C. Dauphin-Villemant and M. B. O'Connor (2003). "Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone." Proc Natl Acad Sci U S A **100**(24): 13773-8.

Pickup, A. T., M. L. Lamka, Q. Sun, M. L. Yip and H. D. Lipshitz (2002). "Control of photoreceptor cell morphology, planar polarity and epithelial integrity during Drosophila eye development." Development **129**(9): 2247-58.

Pozzi, A. G., C. P. Lantos and N. R. Ceballos (1997). "Subcellular localization of 3 beta hydroxysteroid dehydrogenase isomerase in testis of Bufo arenarum H." Gen Comp Endocrinol **106**(3): 400-6.

Pursley, S., M. Ashok and T. G. Wilson (2000). "Intracellular localization and tissue specificity of the Methoprene-tolerant (Met) gene product in Drosophila melanogaster." Insect Biochem Mol Biol **30**(8-9): 839-45.

Radyuk, S. N., V. I. Klichko and W. C. Orr (2000). "Catalase expression in Drosophila melanogaster is responsive to ecdysone and exhibits both transcriptional and post-transcriptional regulation." Arch Insect Biochem Physiol **45**(2): 79-93.

Rauch, P., M. Grebe, K. Carsten, D. Spindler and M. Spindler-Barth (1998). "Ecdysteroid receptor and ultraspiracle from *Chironomus tentans* (Insecta) are phosphoproteins and are regulated differently by molting hormone." Insect Biochem Mol Biol **28**: 265-275.

Reed, B. H., R. Wilk and H. D. Lipshitz (2001). "Downregulation of Jun kinase signaling in the amnioserosa is essential for dorsal closure of the Drosophila embryo." Curr Biol **11**(14): 1098-108.

Rees, H. H. (1995). "Ecdysteroid biosynthesis and inactivation in relation to function." European Journal of Entomology **92**: 9-39.

Richard, D. S., S. W. Applebaum, T. J. Sliter, F. C. Baker, D. A. Schooley, C. C. Reuter, V. C. Henrich and L. I. Gilbert (1989). "Juvenile hormone bisepoxide biosynthesis in vitro by the ring gland of Drosophila melanogaster: a putative juvenile hormone in the higher Diptera." Proc Natl Acad Sci U S A **86**(4): 1421-5.

Riddiford, L. M. (1993). "Hormone receptors and the regulation of insect

metamorphosis." Receptor **3**(3): 203-9.

Riddiford, L. M. (1993). Hormones and *Drosophila* Development. The Development of *Drosophila melanogaster.* M. Bate and A. Martinez Arias. New York, Cold Spring Harbor Laboratory Press. **2:** 899-939.

Riddiford, L. M. (1996). Molecular Aspects of Juvenile Hormone Action in Insect Metamorphosis. Metamorphosis, Academic Press, Inc.: 223-251.

Rizki, T. (1978). The Circulatory System and Associated Cells and Tissues. The Genetics and Biology of Drosophila. M. Ashburner and T. Wright. London, Academic Press: 397-452.

Rizzo, W. B. (1998). "Inherited disorders of fatty alcohol metabolism." Mol Genet Metab **65**(2): 63-73.

Roberts, D. B. (1998). Drosophila. A Practical Approach. Oxford, Oxford University Press.

Romer, F., H. Emmerich and J. Nowock (1974). "Biosynthesis of ecdysones in isolated prothoracic glands and oenocytes of Tenebrio molitor in vitro." J Insect Physiol **20**(10): 1975-87.

Romer, F. and W. Gnatzy (1981). "Arachnid oenocytes: ecdysone synthesis in the legs of harvestmen (Opilionidae)." Cell Tissue Res **216**(2): 449-53.

Rosen, E. D., P. Sarraf, A. E. Troy, G. Bradwin, K. Moore, D. S. Milstone, B. M. Spiegelman and R. M. Mortensen (1999). "PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro." Mol Cell **4**(4): 611-7.

Ruiz de Mena, I., M. A. Fernandez-Moreno, B. Bornstein, L. S. Kaguni and R. Garesse (1999). "Structure and regulated expression of the delta-aminolevulinate synthase gene from Drosophila melanogaster." J Biol Chem **274**(52): 37321-8.

Ruiz de Mena, I., M. A. Fernandez-Moreno, B. Bornstein, L. S. Kaguni and R. Garesse (1999). "Structure and regulated expression of the delta-aminolevulinate synthase gene from Drosophila melanogaster." J Biol Chem **274**(52): 37321-8.

Rulifson, E. J., S. K. Kim and R. Nusse (2002). "Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes." Science **296**(5570): 1118-20.

Rusten, T. E., R. Cantera, J. Urban, G. Technau, F. C. Kafatos and R. Barrio (2001).

"Spalt restricts EGFR mediated induction of chordotonal precursors in the embryonic PNS of *Drosophila*." Development **128**: 711-722.

Ryter, S. W. and R. M. Tyrrell (2000). "The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties." Free Radic Biol Med **28**(2): 289-309.

Sambrook, Fritch and Maniatis (1989). Molecular cloning, a laboratory manual. New York, Cold Spring Harbour Laboratory press.

Sardiello, M., F. Licciulli, D. Catalano, M. Attimonelli and C. Caggese (2003). "MitoDrome: a database of Drosophila melanogaster nuclear genes encoding proteins targeted to the mitochondrion." Nucleic Acids Res **31**(1): 322-4.

Scholz, H., J. Deatrick, A. Klaes and C. Klambt (1993). "Genetic dissection of pointed, a Drosophila gene encoding two ETS- related proteins." Genetics **135**(2): 455-68.

Schubiger, M., A. A. Wade, G. E. Carney, J. W. Truman and M. Bender (1998). "Drosophila EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis." Development **125**(11): 2053-62.

Seegmiller, A. C., I. Dobrosotskaya, J. L. Goldstein, Y. K. Ho, M. S. Brown and R. B. Rawson (2002). "The SREBP pathway in Drosophila: regulation by palmitate, not sterols." Dev Cell **2**(2): 229-38.

Segraves, W. A. and D. S. Hogness (1990). "The E75 ecdysone-inducible gene responsible for the 75B early puff in Drosophila encodes two new members of the steroid receptor superfamily." Genes Dev **4**(2): 204-19.

Sehnal, F. and P. J. Bryant (1993). "Delayed pupariation in *Drosophila* imaginal disc overgrowth mutants is associated with reduced ecdysteroid titer." J. Insect Physiol. **39**: 1051-1059.

Sentry, J. W., M. M. Yang and K. Kaiser (1993). "Conditional cell ablation in Drosophila." Bioessays **15**(7): 491-3.

Shani, N., G. Jimenez-Sanchez, G. Steel, M. Dean and D. Valle (1997). "Identification of a fourth half ABC transporter in the human peroxisomal membrane." Hum Mol Genet **6**(11): 1925-31.

Sharma, R., B. G. Lake, J. Foster and G. G. Gibson (1988). "Microsomal cytochrome P-452 induction and peroxisome proliferation by hypolipidaemic agents in rat

liver. A mechanistic inter-relationship." Biochem Pharmacol **37**(7): 1193-201.

Sharma, R., B. G. Lake and G. G. Gibson (1988). "Co-induction of microsomal cytochrome P-452 and the peroxisomal fatty acid beta-oxidation pathway in the rat by clofibrate and di-(2-ethylhexyl)phthalate. Dose-response studies." Biochem Pharmacol **37**(7): 1203-6.

Sharma, R. K., M. V. Doig, D. F. Lewis and G. G. Gibson (1989). "Role of hepatic and renal cytochrome P-450 IVA1 in the metabolism of lipid substrates." Biochem Pharmacol **38**(20): 3621-9.

Simpson, A. E. (1997). "The cytochrome P450 4 (CYP4) family." Gen Pharmacol **28**(3): 351-9.

Simpson, P., P. Berreur and J. Berreur-Bonnenfant (1980). "The initiation of pupariation in Drosophila: dependence on growth of the imaginal discs." J Embryol Exp Morphol **57**: 155-65.

Snodgrass, R. (1935). Principles of Insect Morphology. New York, McGraw-Hill.

Sondergaard, L. (1993). "Homology between the mammalian liver and the Drosophila fat body." Trends Genet **9**(6): 193.

Sood, V., K. Colleran and M. R. Burge (2000). "Thiazolidinediones: a comparative review of approved uses." Diabetes Technol Ther **2**(3): 429-40.

Stark, W. S., T. N. Lin, D. Brackhahn, J. S. Christianson and G. Y. Sun (1993). "Fatty acids in the lipids of Drosophila heads: effects of visual mutants, carotenoid deprivation and dietary fatty acids." Lipids **28**(4): 345-50.

Stark, W. S., T. N. Lin, D. Brackhahn, J. S. Christianson and G. Y. Sun (1993). "Phospholipids in Drosophila heads: effects of visual mutants and phototransduction manipulations." Lipids **28**(1): 23-8.

Stern, D. (2003). "Body-size control: how an insect knows it has grown enough." Curr Biol **13**(7): R267-9.

Stocker, H. and E. Hafen (2000). "Genetic control of cell size." Curr Opin Genet Dev **10**(5): 529-35.

Strecker, T. R., M. L. Yip and H. D. Lipshitz (1991). "Zygotic genes that mediate torso receptor tyrosine kinase functions in the Drosophila melanogaster embryo." Proc Natl Acad Sci U S A **88**(13): 5824-8.

Studinger, G. and A. Willig (1975). "Biosynthesis of alfa- and beta-ecdysone in isolated abdomens of larvae of *Musca domestica*." J. Insect Physiol. **21**: 1793-

1798.

Sutherland, T. D., G. C. Unnithan, J. F. Andersen, P. H. Evans, M. B. Murataliev, L. Z. Szabo, E. A. Mash, W. S. Bowers and R. Feyereisen (1998). "A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis." Proc Natl Acad Sci U S A **95**(22): 12884-9.

Tafti, M., B. Petit, D. Chollet, E. Neidhart, F. de Bilbao, J. Z. Kiss, P. A. Wood and P. Franken (2003). "Deficiency in short-chain fatty acid beta-oxidation affects theta oscillations during sleep." Nat Genet **34**(3): 320-5.

Talbot, W. S., E. A. Swyryd and D. S. Hogness (1993). "Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms." Cell **73**(7): 1323-37.

Tatar, M., A. Bartke and A. Antebi (2003). "The endocrine regulation of aging by insulin-like signals." Science **299**(5611): 1346-51.

Thomas, H. E., H. G. Stunnenberg and A. F. Stewart (1993). "Heterodimerization of the Drosophila ecdysone receptor with retinoid X receptor and ultraspiracle." Nature **362**(6419): 471-5.

Thorpe, W. (1930). "The Biology, Post-embryonic Development, and Economic Importance of Cryptochaetum iceryae (Diptera, Agromyzidae) parasitic on Icerya purchasi (Coccidae, Monophlebini)." Proc. Zool. Soc. **60**: 929-971.

Thummel, C. S. (1995). "From embryogenesis to metamorphosis: the regulation and function of Drosophila nuclear receptor superfamily members." Cell **83**(6): 871-7.

Thummel, C. S. and V. Pirrotta (1991). "Technical Notes: New pCaSpeR P-element vectors." D. I. N.

Tijet, N., C. Helvig and R. Feyereisen (2001). "The cytochrome P450 gene superfamily in Drosophila melanogaster: annotation, intron-exon organization and phylogeny." Gene **262**(1-2): 189-98.

Tio, M., C. Ma and K. Moses (1994). "spitz, a Drosophila homolog of transforming growth factor-alpha, is required in the founding photoreceptor cells of the compound eye facets." Mech Dev **48**(1): 13-23.

Tomancak, P., A. Beaton, R. Weiszmann, E. Kwan, S. Shu, S. E. Lewis, S. Richards, M. Ashburner, V. Hartenstein, S. E. Celniker and G. M. Rubin (2002). "Systematic determination of patterns of gene expression during Drosophila

embryogenesis." Genome Biol **3**(12): RESEARCH0088.

Tontonoz, P., L. Nagy, J. G. Alvarez, V. A. Thomazy and R. M. Evans (1998). "PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL." Cell **93**(2): 241-52.

Truman, J. W. and L. M. Riddiford (2002). "Endocrine insights into the evolution of metamorphosis in insects." Annu Rev Entomol **47**: 467-500.

Truman, J. W., W. S. Talbot, S. E. Fahrbach and D. S. Hogness (1994). "Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during Drosophila and Manduca development." Development **120**(1): 219-34.

Tsai, S. Y. and M. J. Tsai (1997). "Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age." Endocr Rev **18**(2): 229-40.

Tu, M. P., C. M. Yin and M. Tatar (2002). "Impaired ovarian ecdysone synthesis of Drosophila melanogaster insulin receptor mutants." Aging Cell **1**(2): 158-60.

Uv, A., R. Cantera and C. Samakovlis (2003). "Drosophila tracheal morphogenesis: intricate cellular solutions to basic plumbing problems." Trends Cell Biol **13**(6): 301-9.

van Veldhoven, P. P. and G. P. Mannaerts (1993). "Sphingosine-phosphate lyase." Adv Lipid Res **26**: 69-98.

Venkatesh, K. and G. Hasan (1997). "Disruption of the IP3 receptor gene of Drosophila affects larval metamorphosis and ecdysone release." Curr Biol **7**(7): 500-9.

Voet, D. and J. Voet (1995). Biochemistry, John Wiley & Sons, Inc.

Volk, T. (1992). "A new member of the spectrin superfamily may participate in the formation of embryonic muscle attachments in Drosophila." Development **116**(3): 721-30.

Wagner-Bernholz, J. T., C. Wilson, G. Gibson, R. Schuh and W. J. Gehring (1991). "Identification of target genes of the homeotic gene Antennapedia by enhancer detection." Genes Dev **5**(12B): 2467-80.

Wanders, R. J. (2000). "Peroxisomes, lipid metabolism, and human disease." Cell Biochem Biophys **32**: 89-106.

Wang, S. F., K. Miura, R. J. Miksicek, W. A. Segraves and A. S. Raikhel (1998). "DNA binding and transactivation characteristics of the mosquito ecdysone

receptor-Ultraspiracle complex." J Biol Chem **273**(42): 27531-40.

Warren, J. T., A. Petryk, G. Marques, M. Jarcho, J. P. Parvy, C. Dauphin-Villemant, M. B. O'Connor and L. I. Gilbert (2002). "Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of Drosophila melanogaster." Proc Natl Acad Sci U S A **99**(17): 11043-8.

Waxman, D. J. (1996). "Role of metabolism in the activation of dehydroepiandrosterone as a peroxisome proliferator." J Endocrinol **150**: S129-47.

Weatherbee, S. D., G. Halder, J. Kim, A. Hudson and S. Carroll (1998). "Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the Drosophila haltere." Genes Dev **12**(10): 1474-82.

Weber, U., V. Siegel and M. Mlodzik (1995). "pipsqueak encodes a novel nuclear protein required downstream of seven-up for the development of photoreceptors R3 and R4." Embo J **14**(24): 6247-57.

Weinberger, C. (1996). "A model for farnesoid feedback control in the mevalonate pathway." Trends in Endocrinology and Metabolism **7**: 1-6.

Wharton, K. A., Jr. and S. T. Crews (1993). "CNS midline enhancers of the Drosophila slit and Toll genes." Mech Dev **40**(3): 141-54.

Wheeler, D. E. and H. F. Nijhout (2003). "A perspective for understanding the modes of juvenile hormone action as a lipid signaling system." Bioessays **25**(10): 994-1001.

Wheeler, W. (1892). "Concerning the "Blood-tissue" of the Insecta." Psyche: A Journal of Entomology **6**: 216-258.

Wielowiejski, H. (1886). "Über das Blutgewebe der Insekten." Zeitschr. f. Wissensch. Zoologie **43**: 512-536.

Wigglesworth, V. (1933). "The Physiology of the Cuticle and of Ecdysis in Rhodnius prolixus (Triatomidae, Hemiptera); with special reference to the function of the oenocytes and of the dermal glands." Quart. J. Microscopical Science **76**: 269-318.

Wigglesworth, V. (1970). "Structural lipids in the insect cuticle and the function of oenocytes." Tissue and Cell **2**: 155-179.

Wilk, R., B. H. Reed, U. Tepass and H. D. Lipshitz (2000). "The hindsight gene is

required for epithelial maintenance and differentiation of the tracheal system in Drosophila." Dev Biol **219**(2): 183-96.

Willnow, T. E. (1999). "The low-density lipoprotein receptor gene family: multiple roles in lipid metabolism." J Mol Med **77**(3): 306-15.

Wilson, T. G. and M. Ashok (1998). "Insecticide resistance resulting from an absence of target-site gene product." Proc Natl Acad Sci U S A **95**(24): 14040-4.

Wolfe, L. (1954). "The deposition of the third instar larval cuticle of Calliphora erythrocephala." Q. Jl microsc. Sci. **95**: 49-66.

Xu, Y., F. Fang, Y. Chu, D. Jones and G. Jones (2002). "Activation of transcription through the ligand-binding pocket of the orphan nuclear receptor ultraspiracle." Eur J Biochem **269**(24): 6026-36.

Yajima, M., M. Takada, N. Takahashi, H. Kikuchi, S. Natori, Y. Oshima and S. Kurata (2003). "A newly established in vitro culture using transgenic Drosophila reveals functional coupling between the phospholipase A2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity." Biochem J **371**(Pt 1): 205-10.

Yamada, M., T. Murata, S. Hirose, G. Lavorgna, E. Suzuki and H. Ueda (2000). "Temporally restricted expression of transcription factor betaFTZ-F1: significance for embryogenesis, molting and metamorphosis in Drosophila melanogaster." Development **127**(23): 5083-92.

Yamamoto, D., E. Kusunose, K. Ogita, M. Kaku, K. Ichihara and M. Kusunose (1984). "Role of cytochrome P450 in co-hydroxylation of prostaglandins." J. Biochem.(Tokyo) **96**: 593-603.

Yamamoto, S., E. Kusunose, K. Ogita, M. Kaku, K. Ichihara and M. Kusunose (1984). "Isolation of cytochrome P-450 highly active in prostaglandin omega-hydroxylation from lung microsomes of rabbits treated with progesterone." J Biochem (Tokyo) **96**(3): 593-603.

Yao, T. P., B. M. Forman, Z. Jiang, L. Cherbas, J. D. Chen, M. McKeown, P. Cherbas and R. M. Evans (1993). "Functional ecdysone receptor is the product of EcR and Ultraspiracle genes." Nature **366**(6454): 476-9.

Yip, M. L., M. L. Lamka and H. D. Lipshitz (1997). "Control of germ-band retraction in Drosophila by the zinc-finger protein HINDSIGHT." Development

124(11): 2129-41.

Yoshioka, T., H. Inoue, T. Kasama, Y. Seyama, S. Nakashima, Y. Nozawa and Y. Hotta (1985). "Evidence that arachidonic acid is deficient in phosphatidylinositol of Drosophila heads." J Biochem (Tokyo) **98**(3): 657-62.

Yun, C. H., C. M. Tse, S. K. Nath, S. A. Levine, S. R. Brant and M. Donowitz (1995). "Mammalian Na+/H+ exchanger gene family: structure and function studies." Am J Physiol **269**(1 Pt 1): G1-11.

Zhang, K., M. Kniazeva, M. Han, W. Li, Z. Yu, Z. Yang, Y. Li, M. L. Metzker, R. Allikmets, D. J. Zack, L. E. Kakuk, P. S. Lagali, P. W. Wong, I. M. MacDonald, P. A. Sieving, D. J. Figueroa, C. P. Austin, R. J. Gould, R. Ayyagari and K. Petrukhin (2001). "A 5-bp deletion in ELOVL4 is associated with two related forms of autosomal dominant macular dystrophy." Nat Genet **27**(1): 89-93.

Zhang, Q., C. Ragnauth, M. J. Greener, C. M. Shanahan and R. G. Roberts (2002). "The nesprins are giant actin-binding proteins, orthologous to Drosophila melanogaster muscle protein MSP-300." Genomics **80**(5): 473-81.

Zhong, W., F. M. Sladek and J. E. Darnell, Jr. (1993). "The expression pattern of a Drosophila homolog to the mouse transcription factor HNF-4 suggests a determinative role in gut formation." Embo J **12**(2): 537-44.

Zhou, D., J. D. Lambert, K. P. White and G. G. Haddad (2003). Gene Expression in Response to Long-term Hypoxic Environment in Drosophila melanogaster. A. Dros. Res. Conf. 44.

Zinke, I., C. Kirchner, L. C. Chao, M. T. Tetzlaff and M. J. Pankratz (1999). "Suppression of food intake and growth by amino acids in Drosophila: the role of pumpless, a fat body expressed gene with homology to vertebrate glycine cleavage system." Development **126**(23): 5275-84.

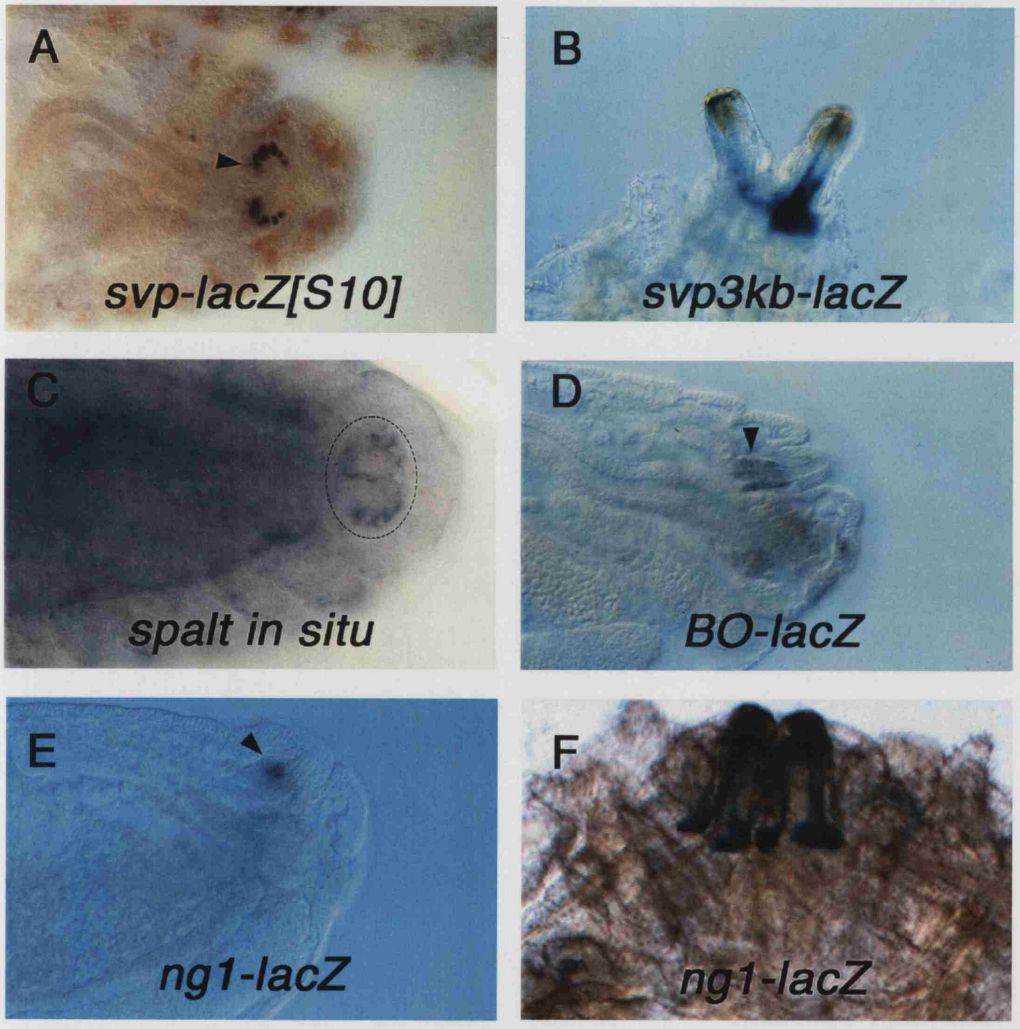
Zitnan, D., T. G. Kingan, J. L. Hermesman and M. E. Adams (1996). "Identification of ecdysis-triggering hormone from an epitracheal endocrine system." Science **271**(5245): 88-91.

zur Lage, P., Y. N. Jan and A. P. Jarman (1997). "Requirement for EGF receptor signalling in neural recruitment during formation of Drosophila chordotonal sense organ clusters." Curr Biol **7**(3): 166-75.

APPENDICES

Appendix 1. A Subset of Oenocyte Markers Labels an 8th Abdominal Cluster that Differentiates into the Posterior Spiracular Glands

Previously it was noted that A1-A8 give rise to *svp-lacZ*-positive clusters of cells that delaminate from the ectoderm (P. Elstob and A. Gould, unpublished). The clusters in A1-A7 differentiate into oenocytes, while the 8th cluster becomes associated with the posterior spiracles (Appendix Figure 1A-B). I find that embryonic expression of two other oenocyte markers also labels this A8-derived cluster of cells, *spalt* (Appendix Figure 1C-1D) and *ng1-lacZ* (Appendix Figure 1E). Both markers also label cells that differentiate into the larval posterior spiracular glands (Appendix Figure 1B and 1F). The observation that oenocytes and spiracular glands appear to share a common ectodermal origin and several molecular markers suggests that they might be serial homolgues. In addition, there are intriguing parallels between lipid-processing by oenocytes and the postulated role of spiracular glands. The latter are believed to secrete lipophilic substances to the external spiracular surface, a process believed to keep the spiracles dry, even when larvae are buried in the food .


A
svp-lacZ[S10]
B
svp3kb-lacZ
C
spalt in situ
D
BO-lacZ
E
ng1-lacZ
F
ng1-lacZ

Appendix 2. P-Insertion Sequences Recovered by Inverse PCR

Sequences adjacent to one or both ends of each P-element were obtained by inverse PCR (Section 2.2). The raw data for each of the sequences recovered is presented.

56B 5'end with kp53 primer:

NNGGNNGNTTNNTNTAANCCGTCCCTTATGTNATTTATCATGGTGTGCTCTGTGTACGAGCCCCTTTTTGCTTTGAAAAC
TGTTACAGGCGAAACGCGAGCATTGCCAGGTAGAATTTACCCGTGTCCTAGGAGGGTTTTCAAACAAAATAAATGTAATC
AATTGACGACTTAATTGAGTTACATGCATTCACTGGTGGTGATTAAGAGGATTTTGAAATGCCTGCTTTTCGTGACTCAT
CTTTGTTATCTCCCAGTTTCTTGGGCCTGGCAATGCTCCCCGTCAACTGTTGAGCTCCCATACTCTCCTGCTTTCGCACT
ATGCCTTTCGTTTATTAATGAAATCCGTGTCTGCTTAAAGTCAGACTTCCAAAGTTAATGCAAATATATGCGGCCAGGTC
TTAGATTTCAGCTTGAGGGGCGTCTGCCAGGTTTCGAGCCAGTTTTAAAGTATTTTTTCAGCCGATTATTCACAGACTTC
CGTGGCAACAACTACGTCAGCTACTCAGACACGGCTTTGGGGTGTGTCTGTGAGTGTGTCGGTTGGTGTGCGCTGTGCAC
GCGGCTGGGGCATGCACAGCCCATGAATAGACAGCCCGATGGAGCCCCACACCCTNCCCAGCTNCTNCTCTACTGACCCG
TTCTCTCTACACCAAACACAAGACCCCGCANTGAACAGCTGATGACGTCCATGCGTTGATTACGTGCTNTGNGGCCTTTT
TCTACTGGCTGGGGGCTGNGTCCTGCACANAAAGAAATCNNTTTGGNGATTTTTTGGGTCTTNCCNGAAAAGATNCCNCC
TTTTAAAATNGCCNTNCNCTTTTAAAGAGTTANTNAAAACNANTTGGNTNNCNNNANNTTTTTTAACTTNTTGCCACCNA
NNTTNNNNTN

56B 5'end with T7 primer:

GNNGGNNNGNNTNNNNTTGATTGATTTAGCGGCCGCGAATTCGCCCTTCTGCAGAAGCTTCAAGCCTCCTGAAAGATGAA
GCTACTGTCTTCTATCGAACAAGCATGCGATATTTGCCGACTTAAAAAGCTCAAGTGCTCCAAAGAAAAACCGAAGTGCG
CCAAGTGTCTGAAGAACAACTGGGAGTGTCGCTACTCTCCCAAAACCAAAAGGTCTCCGCTGACTAGGGCACATCTGACA
GAAGTGGAATCAAGGCTAGAAAGACTGGAACAGCTATTTCTACTGATTTTTCCTCGAGAAGACCTTGACATGATTTTGAA
AATGGATTCTTTACAGGATATAAAAGCATTGTTAACAGGATTATTTGTACAAGATAATGTGAATAAAGATGCCGTCACAG
ATAGATTGGCTTCAGTGGAGACTGATATGCCTCTAACATTGAGACAGCATAGAATAAGTGCGACATCATCATCGGAAGAG
AGTAGTAACAAAGGTCAAAGACAGTTGACTGTATCGATTGACTCGGCAGCTCATCATGATAACTCCACAATTCCGTTGGA
TTTTATGCCCAGGGATGCTCTTCATGGATTTGATTGGTCTGAAGAGGATGACATGTCGGATGGCTTGCCCTTCCTGAAAA
CNGACCCCAACAATAATGGGTTCTTTGGCGACGGGTCTCTCTTATGNATTCTTCGATCTATTGGNTTTAACCGGACAAAT
ANCCCGNAAGAATGACTTNTNTGTNACTCATTTTAACTGTATGTTGGTGGCANTANNTAAAANAAATNNGNGACNCAAAT
NGNTTTNGNAACTNTNNAAANNGNTNGGTNTTNNAANGNGGTATNTTTNNCGGANNNCCAAAAATNCCNNANCGNTTNTT
TT

56B 3'end with T7 primer:

GNNGGNAGNNTTNNTTTGATTGCTTTNGCGGCCGCGAATTCGCCCTTCCTTAGCATGTCCGTGGGGTTTGAATTAACTCA
TAATATTAATTAGACGAAATTATTTTTAAAGTTTTATTTTTAATAATTTGCGAGTGCGCAAAGCTCTAGCTAGAGGACCC
AGATCCACTAGTGGCCTATGCGGCCGGTGGGGAGCTTCAACTGCGCCGCAGTTTGCATTCAAAATGCGGGGGGTGGGGCG
CAATGGAGAGGGGTGCGGTAACGGTATATCGGCCCATTTACACGCCCACATTCGCGAGAGAGTGGAGGCCATCGCTTCAA
GTTCCGCGCAAAATTTACACACGAATTCCGATTCCAATCACAAACACGAAGCAAAGACAAAAACAAAAACGCAAGTACTG
CAAACCCCCACACGCACACAAGCGCACAGATAGGCACGCCACAGACTGCTGGCATTTTCGCCTCGTCTCACTCGCACTCA
CTCACTCGTCCGCTCAGGCAAAGTATCAACAAATGTTCACGTGAACATCATGAGGTCAACGCGTGATACCAGACGGACCC
ACACAGACACTCACGCACACCATAAAACACGCAGGCGAGCTGTTCAGCCGTCAAGAAGGTTATTTTTCGAGGGGTCCACT
TGGGCATACCCTCTAAAAGCGGCGATAAAGCAGTATGAGACGGATGGAATGACATTAATTTAGTAACGACAAATATAAAA
TTCCTTTATAGGGAATGTGAAGAAAAATAAAACTTAAACTTCTCAGAGATCTGCATCTAATCTAGCTGCTCCATGCTACC
CCTGAATACCCTACACGGTCTGAACAGTCGCCCCACAAGTTTGGTGGTGGGGTATGGCGTGCTAGTTATTCGTTGTTGTT
GCGCAAGANAGCGCCAATAACCACAGCTG

56B 3'end with pyr2 primer:

GAGGNAGNTTNNTTTAATCCCNTTGGGACCCACCCAAGAGCGAGCGGCAGAAACAGCTGTGGTTATTGGCGCTCTCTTGC
GCAACAACAACGAATAACTAGCACGCCATACCCCACACCAAACTTGTGGGGCGACTGTTCAGACCGTGTAGGGTATTCAG
GGGTAGCATGGGAGCAGCTAGATTAGATGCAGATCTCTGAGAAGTTTAAGTTTTATTTTTCTTCACATTCCCTATAAAGG
AATTTTATATTTGTCGTTACTAAATTAATGTCATTCCATCCGTCTCATACTGCTTTATCGCCGCTTTTAGAGGGTATGCC
CAAGTGGACCCCTCGAAAAATAACCTTCTTGACGGCTGAACAGCTCGCCTGCGTGTTTTATGGTGTGCGTGAGTGTCTGT
GTGGGTCCGTCTGGTATCACGCGTTGACCTCATGATGTTCACGTGAACATTTGTTGATACTTTGCCTGAGCGGACGAGTG
AGTGAGTGCGAGTGAGACGAGGCGAAAATGCCAGCAGTCTGTGGCGTGCCTATCTGTGCGCTTGTGTGCGTGTGGGGGTT
TGCAGTACTTGCGTTTTTGTTTTTGTCTTTGCTTCGTGTTTGTGATTGGAATCGGAATTCGTGTGTAAATTTTGCGCGGA
ACTTGAAGCGATGGCCTCCACTCTCTCGCGAATGTGGGCGTGTAAATGGGCCGATATACCGTTACCGCACCCCTNTTCAT
TGCGCCCCACCCCCGCATTTTGAATGCAACTGCGGCGCAATTGAAGCTCCCACCCGGNCCGCATANGCCANTTAGNGGAT
CTGGGTCCTTAGCTAGAGCTTGCGCCTCGCAAATATTAAAAATAAAACTTTAAAAATAATTTCGCTAATTAATATTATGA
AGTCAATCN

P0206 5'end with Kp53 primer:

GNGGGNNNNNAGGGGTTTTNNNTTTGAANCCCTTTCANCTTATNNTATTTCATCATGGNTNGGGTCATGTGGCGGGCAAC
TAATCGGTTTCCGATGGCGGGGAATAAAGCGCCCCAGGACAGGGGTTACCTAATCGTATGACCACTTGTCGTTTAGAATT
AAATGTACCAAATGGCAAAAAACTTAACCTTTTTTCTTGTTAAGTTCAAATGAAATTACTGTGTATATTGTATATTAATA
TTTACTTTTTTTTGAAGCAAAACTGCAGTTGTTGGTTGGCATAGCATTTAATAAAATTCAAATACAATTATTCGAAAAAA
TTGCTCATAGCAAATAAAGTGTATTTTCCTTGGCAAGGTTCGTTCCTGGTATATCAGAGGGGAATCCCTGACTGTCAGCT
GTGACCTTGCCTCACCCGGAAAGTTTCCCACCCACAGCGCGGGGGTTTTCCGCCCGCATCAGCTGTTGCTCTGCCACACA
CTGACACACTGCCGGCACATTCCCTCACGCAATGGGATTTTTGCCATAGCAACCGGGTCCATCAATCATTATATTTGTCC
CTGAAATCAAACGCAAAAAGCATCCTGTTGAACCGGCGAACAGCAACAGGACGAAGGCGACATTTCCGGTTGCGCATTCC
GAAGAGCGAGATGGCCTCAGAACGGCCGAACGAGAGTGAAAACGAGGTCAGGGTAAAATATATTTTAAAGAGTCCTTGAG
ACAGCATATAATTCGAAAAGGACCTCATCGCCAAAGAAAGAAGATGAATTANCCAAAAGAATGAAAAGGGAAAANANANA
ACCCCCCCCACCTGCTTGTTGTTATGGCAACCAAGGGTTGGATGCCTTCCTTTTTTTNTGGNTTNTTTCGCATTCCCGGCA
ACGCATGG

P0206 3'end with Pyr2 primer:

GGGGTTNNTCNNATCCTTGGATGACCCAACCGACCCAAATAATTGTAATTTATATGCCAAATGCCGAAAATAAAATCGAA
ACTATAATAAATGACTGACCGGCATTAATGAAAACAAAAAATCCGCTGCTGGCCGGTGGAAACCGAGCTGGGACTAATGT
GAAATGCCTCGAAGAGATTTGTCCAGATAACCAGATTCCGTTTTTCCAGTTGCCAAATGTTCGTTTTCTTTTTTCATTAT
TTGGTATATTACCGCACACGCCCTCGCAGTGACCACGCCCACGTGGCAGCACCTCCGCCTCCATTCTCTTTATTAGAGTG
GACTGCGAAGAATTTTGGGGAGGGAGCCGTCGAATGGTAGCCCTGGTTTATCTATCACCCACCTGGAAAGGTCAAAGTTG
GGGGCTCCCCTGCCCCCATCGATTCCCCGGCCGACAATCCTTGGTGATCCTCTAGCTAGAGCTTTGCGTACTCGCAAATT
ATTAAAAATAAAACTTTAAAAATAATTTCGTCTAATTAATATTATGAGTTAATTCAAACCCCACGGACATGCTAAGGAAG
GGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTG
TTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGGCC
TAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGGCCGTGCCN

P890 5'end with kp53 primer:

GGAGTTTNNNCNATCCCTTTGANCNCCNNATTTATTATGCTCTGACCAAGAGCTCCATCTCTCTCGGCTCCACTGCCATA
TCATTAACTGTATCGGAATTTCGCTCGTTTGCCTTTTTTCTGCCCCATAAAGGCGGCCGAAAAGGAACAAAAAGCATTTG
GCTGGCTGGGTTTTTATTAACACCGCTAGATGTCTCTCTCTGGCAGCCCCGTTTAGGGTCTAATTCAATTGGCAGCGTCT
CTCTTTCTCGGGCCATATGACTTATGTGTGTGGGGTCTCTGTGGCGATGATCTCCCGATCCCCCGAACATCCTGATGGCT
CCTTTTCTGTGAACCTAATCCTGTGCTCCTCGCCACCTCTTGTTAGTGTGCCTCGCACTCGCATCCATGTTGGAGGTAAC
CCCATCTCGAAACCCAGCCCATCCCTAATCCTCAATCCACTGGTTACATGGACTTTGCGGAGTTCGCGCAGTCAGCCGGA
AACCAGGCAAAGCGCCATTCGCCATCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCNTATTACGCC
AGCTGGCNAAAGGGGGATGTGCTGNAAGGNGATTAANTGGGTNACNNCANGGNTTTCNAGT

P890 3'end with Pyr2 primer:

GGGGTTNNTCTNATCCTTTGATGAGCCCTACGTCATCTTCGGCATTGTAGTTGTAATTCGCAAAGCAAGGAGCGCGCAAC
GAGAAACGCGCGCGAAAAAGAAATAAATAAATAAATAGCCAAGGAGAAGAGTGTGTGCGCCAAAAGGAGTGAGTGAGTGG
GTTGTGAAGGGGGCGTGCGGCAGGAGTGCAAAATAAACGAGTTGCCACAGCAACAACAACGAGATTCGCAAAAAAGGGGG
AAAATTGACGCATTCCAAACGCGCGATTTGGCTTACAAGTATTGTTTTTTTTCCCGAGCGGTGTTGTTGTTGGTGTTTCT
GTTTTTGCTAGTTGATTTTTTTCGTGCTAATACCGGTCTTTCTTAGTCGGTCTCTCTTACCCGAACTTTGTGCCTCTCTC
TTATCCACAGTGAGTTTTCCACAAGTCGAGAAATCAAACAATTCGTGGAAATAAAAAAAATAAACTGCAAGTCCCAGTAT
AAATCGAACAAAAACGAGGAAAATCCAATCTAAAGGCAGGCAAATGCAAATGCAAAGGCAAACGAAATCGCACTACTATT
ATGGGCCGCTCCAAGTTTCCCGGCAAACCCTTCAAGTCGATCAACCGTAAGCGGATCCGTCGAGCCGTATAACCATCTGT
CAAAAAAGGATTTCCTTTGCCCAGTCGTACGACTTTTGTACAGATGGTTATCAAATGTGGACATAAAAAAGAGGATGTTT
GGATGTGGTCATAGACCTAATGGGACAGTGATGGAGTTGAATGACNCCCACAAGCTTTGCGTACTCGCNAATTATTNAAA
AA

P1785 5'end with kp53 primer:

GNGNNGTNTTNCNCGTATCCCTTTGAANCTTCCNNATTTCATTATGCTTGCGCGCGTGCCTGGATAACAGTGGTCAAAAA
TATCGCACGCACTTTCAACATCGATGACAACCATTTACGCATCTTACTCATCACTAAACTTAAAGGAAACGCGCAAGTCT
GGTTACATGCGCACCCTGCTCGATTGATCGAACCAATTGACAATTTGCTTGATCAATTGTCATTGACTTTTGGCGAGCAA
TCATCCAAGGCTGAGATCCGGAAACCAGGCAAAGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGG
TGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTT
TCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGGCTGCTGCCTAAACGACGCATTTCGTACTCCAAAGTACGA
ATTTTTTCCCTCAAGCTCTTATTTTCATTAAACAATGAACAGGACCTAACGCACAGTAAGGGCGAATTCGTTTAAACCTG
CAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTA
TCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCA
CATTAATTGCGTTGCGCTCACTGGCCCGCTTTNCAGTCGGGAAACCTGTCGTGCCAGCTTGCATTAATGAATCGGCCAAC
GCGG

P1785 3'end with Pyr2 primer:

GGNGGTTNCNCTATCCCTTTGAAGCGCCCAGNNNNTTATCGTAATCGGTTATCGTCTCTTTGGCGAGAGCGAGAGCATCA
ACTGGAAAACGCTGGCTCTCCGCTCTCTCGTTGGATCGCTGCGGCGAGTGAAATGTTGTGTTCTCTTTTGTGGCGCCAGC
CTGCGTAAACTGCGCCGTCTGGATAACGGAACTAACTGTAGCCTTGCTTTGCTCTGGTTGTTGTTGTTCTTGGAACTGCA
AGCTCGATCGTGTTCCGTTAAGCATTGCCTTCGCTTCTGCTAGCTCTGCGCGTAGTTGCTGTAGCTCGAACATGTTTGTC
TCCCTCTCACTGCCAGTTTCGGTTGATTCTTTTCTGTTCATCGCATTCACACTTACGTGATCGGTGTTAATTTCACAATT
CTGAAAATCCAGTGAAGAAAAAATGTCGTTTCCTGGTGGAGCGTTTTTCTGGGGGTGCTCCGGTGGGAATGTGCGGATTA
GAGGGCCTGGCTATCTGGCAGACACAAATGCCCATGACGATCCCAGCTCACCCACATCCGAGTAGCACTGCTCCCATGCG
CGATTGGCAAGTGCACCATCTTGATCGGGCGATAAGGGGCCACCAGCAAACAACGAGGCTTCACATGGGGGTCAATTGTT
ATCACTATATGCACAGCAAATACTATATACATTCGACACATTCAATTGAGCAAGAAGGCAAATTCCATCGAATGCCAGTT
AAACGCAATTAGGGCAATAAAACAATAAAAGGGAATCGATATAGTAGGAGTGGGCCCGACTTTTAAGGAAAGACACGC

BI-11963 with 5'end with kp53 primer:

GGGGTTAAAAGAGATCNCTTTGACCCATCATGAAATAACATAACATCCGTTAACATCCCGTTTTTCCCGATTTGGCTACA
TGACATCAACCATATCAGCAAAAGTGATACGGGTATTATTTTTGCCGCTATTTCTCTGTTCTCGCTATTATTCCAACCGC
TGTTTGGTCTGCTTTCTGACAAACTCGGGCTGCGCAAATACCTGCTGTGGATTATTACCGGAAACCAGGCAAAGCGCCAT
TCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAACGACGGNCANTGCCAAGCT
CTGNTGNTNTAAACNATNCATNTNGTACTCCAAA

BI-11963 3'end with Pyr2 primer:

GAGNGTTACNACNGATCTCTTTGAAGGGCCACAAAATGTTATNCAATCGTGGCNGNGCTGCCTCCGNGTGAAAAAAAATG
AGAAAAACCAGACTGCTTTTTTTCTTCTCTTTTCTCTTTTCATTTCAAATNTTACGCTCTCGCTCTCGCGGACAGCGAC
NCCGCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTNCAGGGGGAAACGCCTGGTATCTTTATAGTC
CTGTCGGGTTTCGCCACCTNTGACTTGAGCGTNGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCC
AGCAACGCANGCTTCTAGAGATCTGCAGGTCGACGGATCCGTCGNGCGTATAACCATCTGTACAAAAAAGGATTTCCTTT
GCCCAGTCGNACGACTTTGTACAGATGGTTNCCAGATGTGGACATAAAAAGAGGATGTTTGGATGTGGTCATAGACCTAA

TGGACAGTGATGGANTTGATGACGCCGACAAGCTTTGCGTACTCGCAAATTATTAAAAATAAAACTTTAAAAATAATTTC
NGTCTAATTAATATTATGAGTTNAATNAAACCCCACGGACATGCTAANGAAGGGCGAATTCTCGGCCGCTAAATTCAATT
CGCCCTATAGTGAGTCGTATTACAATTNACTGGCCGGACGNTTTACAACGTCNTGACTGGNAAAACCCTGGCGTTTCCCA
CTTAATCGCCTTGCACACATCCNCCTTTCGCCANCTGGCGTAATATCNAAAAAGGCNCNCCGANCGCCTTTCCAACAGTN
GCGCAAGCTAT

BI-12139 5'end with kp53 primer:

GGGNGTNTTNNTCGTNATCCCTTTGGANCTTCCGTTATTTCATTATGGGCAGTGTAGTGAAAAAAGCGACAATAAAATCG
CCGATTGCACGCCTTCTGCTCATTACTTCGCGTTCCGCTCTCAAAGCGTAAGCACTTTTCTTTTATTTCTACGACGCACA
AGCCCACTGAATATTTTCTTTATTCTTCCGGAAAAACAAAACCGCATACACACAGAAATACCCCCACAACAACAACAACA
ACAACCATGTCTCTGCAGTCGGACGCCGTTTTCCAAAAGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAG
GGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCC
AAGCTCTGCTGCTCTAAACGACGCATTTCGTACTCCAAAGTACGAATTTTTCCCTCAAGCTCTTATTTTCATTAAACAAT
GAACAGGACCTAACGCACAGTAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGC
TTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAG
CATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGT
CGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTN
CGCT

BI-12139 3'end with Pyr2 primer:

GGGGGTTNNNCTATCNCTTTGANGACACNGCCCCTGTTGTGTGGGAGCGGGATGGTCGTATAGCAAGTTGCCGGTCGCGA
TATGTCGCCCTCACTCGCACCTGTTGCATTCGCATAAGCCTTGTTGTGCTGTTATTGTCGCGCTCCCTCTCTCTCTCGCT
CTCTTCCAAATGTTAGCCAGCTGATGCACAGTGGGCATGAACTCAAAATAAAGTGGCTGCACGAAAACCTTTTGCATATC
AGATATAGCTTTTATTTATATCGTTTATGCGATATTTCTTTCATTAAGCTGAATTGAAATTCTGATATTTGATGATATCA
GAATGAAAAAGTAAATAACTGTATCGATATCATTTGGTTTTCACCGCTTGTTAGCCCACTGTTACAGGTGTGGAGTTTAG
ACTTTAACATTAACATGAAACGCCTAACATGAAGCCGTAACAGCGGTTATCAGCTGTTGTCCACGATTTAGTGCAAAGTG
CGCACCCAGTTTGAAATGCCCATAAAACGGCGATAAGGCTTGAAGACTCTTCAGATTATTTCGACAAAATATGTCACCTA
ACGAGCCCATTGAGAAAATATTTTTAACCCCCCAAATGCCATATATCGTGATTCGATTCATAAAACGCAAGCATTATTCA
GTACTATTATTTTGCATTGACCAATGGATTTCAAGTTAGTAAATGCATCAATTTATANGGGTTAAACGACAAGTAGAATG
TGCCGTTCGTATCAACTTCACTTCCTTATGAGCTCTCGAACTTTTCCNGGATCCGTCGAGCCGTATAACCATCTGTCAAA
AA

BI-12247 3'end with Pyr2 primer:

GGNGTACNATATCNCTTTGANGCTCCNGCTAAATTNATTTCTATCGTACCAGACTGCCGCGCTCTCTGCGCTCTCACTGC
GCATGCTCACTGCTGCTCTCTTTGCTGGAAGTGGTGAGAATAGGAGCTTCAACAAAAATCTTCTTTGACATTTGGATCAC
ATTTAGATCACATTTGGATTCATCACGATTTGTTAATACTCGAAGGTTTTCAAATAGTACGCAGTGCATTTAAAATGATC
ATTGTTTCTTTGCCTCTCCCGCTCACTGGTTCACATAACAGCATCAGTTTGCCGGTAAGCGGCAGGGTCGGAACAGGAGA
GCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTC
GATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCAAGCTTCTAGAGATCTGCAGGTCGA
CGGATCCGTCGAGCGTATAACCATCTGTACAAAAAAGGATTTCCTTTGCCCAGTCGTACGACTTTGTACAGATGGTTATC
AGATGTGGACATAAAAAGAGGATGTTTGGATGTGGTCATAGACCTAATGGACAGTGATGGAGTTGATGACGCCGACAAGC
TTTGCGTACTCGCAAATTATTAAAAATAAAACTTTAAAAATAATTTCGTCTAATTAATATTATGAGTTAATTCAAACCCC
ACGGACATGCTAAGGAAGGGCGAATTCCCCGGCCCGCTAAATCAATTCGCCCTATAGTGAGTCGTATTACAATTCN

L14A2 5'end with kp53 primer:

GGGNGTTTNCAANAANTCCCTTTGNANCTTCCNTTATTTCATTATGGCCCCGACGAAGCGACTACGAAAGCTCTGGATCT
GCCGCTGGACTACGTGGGTCTGGCCATTCTCATCGTGAGCTTCCGGGTGCTCGCATATCTGGCTCTAAGACTTCGGGCCC
GACGCAAGGAGTAGCCGACATATATCCGAAATAACTGCTTGTTTTTTTTTTTTTTACCATTATTACCATCGTGTTTACTG
TTTATTGCCCCCTCAAAAAGCTAATGTAATTATATTTGTGCCAATAAAAACAAGATATGACCTATAGAATACAAGTATTT
CCCCTTCGAACATCCCCACAAGTAGACTTTGGATTTGTCTTCTAACCAAAAGACTTACACACCTGCATACCTTACATCAA
AAACTCGTTTATCGCTACATAAAACACCGGGATATATTTTTTATATACATACTTTTCAAATCGCGCGCCCTCTTCATAAT
TCACCTCCACCACACCACGTTTCGTAGTTGCTCTTTCGCTGTCTCCCACCCGCTCTCCGCAACACATTCACCTTTTGTTC
GACGACCTTGGAGCGACTGTCGTTAGTTCCGCGCGATTCGGTTCGCTCAAATGGTTCCGAGTGGTTCATTTCGTCTCAAT
AGAAATTAGTAATAAATATTTGTATGTACAATTTATTTGCTCCAATATATTTGTATATATTTCCCTCACAGCTATATTTA
TTCTAATTTAATATTATGACTTTTTAAGGTAATTTTTTGTGACCTGTTCGGAGTGATTAGCGTTACAATTTGAACTGAAA
GTG

L14A2 3'end with Pyr2 primer:

GGGGTTNAAANAATCNCTTTGAAGGNCCGGCCGNCTTTCTATATCTACAACAAATAATATTAAGACAGTTAGTACGTGTT
GAGAACATTATTTGAGGTTTAGTCAGTGTTTTGCACTAGGCGGTAATAATACAGGCGCCGCAGGCTCACACCCTTTTACC
TTTTTACTAACAATAAAAAAAGAAAATAAATAAGACAAGCCGGCTTAAACCAATCCAACTTGCCAATCAAAACATTATTC
AAGAGCACCTGTGCGGCACTGGGAAAGGATTCGCGCAAAGAACTGTGATATTTTTAAACAATTCTACACGCCGCCCAATT
TGTATTTATTTATTTAATCCGCTCGCATGTAAATATATATATACATACAAAATATATATATATATATTGTATCGAACCAA
TTGCCGACGATTCGGTTCGCTTCGGTTTTTTTTCTGGGCAGTGCGGTGTTTTCCCTCGCTCCTTAACTTTCATCGCTTCA
GCTATTTTCGGTTTTAATTTGTGCAAATATTTAACTTTACTTAAGTGTGTCTCTGGCCCCTCACCTTAGCTGGCTTCAAA
ATAAAATCGACAACAACAAAAAAAACAAAACAGAGAGAATTATACAAAAAATGTTCACCAACAATAACAACAAAGTCGGC
AACAGCGACAAAAGCAGCAACCGTTTGTGAAAAACTCAATTTGCCCAAGAGTGTGAAGGATTTTCAAAATATTTACCCTC
GTCTACGATCCGACCATGGTCCTCGGAGACTAGTTCGTCCGCTCTAGCCCTGCAGCCAAGCTTTGCGTACTCGCAAAATA
TT

OK72 5'end with kp53 primer:

GNNNNNNNNNGGNGNNTNTCTGTCNCTTCCCCTTCGTTATTTATCATGTNNCAGTGTNTGTTTGCTTTTTCCTCTCCAA
CTGGTCGAACAACAACATTAAGTGTAAGAAAACAACAAATGCACACCCATAAACGGAGAGATAAGAACTTNTGGCNGTTG
TATNTTTNACTTAAACTNCTCTNGGNCGAGCNACTGCTCTTATTTNGAGTTGGATNNTCAAGCGGTGAGGATATGTTTCA
GGTGTATAATATCGGAATTGTCGGTGTACCGTGATTAACTGNCTGNNAATCTGTAAACAAAGNGAACAATCATGCTGTTN
GGAGTGCAATCATGAAANNGTCTATTCAAATTGTAAATGTTNGCCAATCAATTACCTTTTAGAGAATNCCCTGCATATGA

AAACTANTANGGCANATAGTGGAAAATCTGNGGGAACCACCCACTGCGAACGCTNNGNGATAATAACANTATTTATACAC
CTATTAAAATGGGGATTGANAGAATATTTTTTTCANTAATTATTGCNAATAATTCCATTCTATTCAATCTTCAAAAACAA
GTCTTCCTTAAAATCGACTGTTCGAGCACTTTTATTGNTGCTTATTNAANGTAACTACTNTCNTCCGTCGATGCCTATNN
TCCGCTNTACTNGNATTANTATTGGCTCTCTNTCTGCGTCTCCCCAAATGATTTTNTCACACACGCNAACAGGCNCTCAT
AACGANGTCCAAATNANNGCNCGGTTTANNGCCAATAGATCGTANAATNCTTAAAGAGAGNAACCGTCGNCAAANAANCC
ATTATNGCTNGGNGCCCGTTTTCNGGAAAGGGCANNCCACCCGG

OK72 3'end with Pyr2 primer:

GNNNNNNGGNNNGGNGGNTNGCCTNTCNCTTTGANGCACCGAAACAGCATCGAATAAAAAAGGTAAATAAGAAACCATAA
TACTGGTTTGCTAACATTTCGCGCATAAAACTTTCTCGCCCCACACTCGCCATCTCTCTCGCCCACCGCAGCCGTTTCGC
TCGCACACTCCGCGGGAGGGGGAAACAGGCTGCGGTGTAATTTTTATATTTTTAGCCAAGTCATCGTTAGAGTTCACTTT
TTTTCCGACCGCGTCATCGTTAGTATTGCAAAAAAATTTTGATGCCTCACACTGAGGTGGGCAAATCGTAATCTGAGATT
AGAAAATATCGGATTCTTGATTCGGAAAATAGTATTATTTGGATGATGCGCTGATAAAATGAAAGAGCCGAAAACAGCAA
TCAATAACATACATATGTACATACATACAGGGATTTCACGTTTCTCGTGAAAACAAACAAACGTGAACACCTGACCATCA
TATAAAAGCAAAGCAGCACTTTTATCTATAAATACTCATATGTGTTCTCTAAAAATTTATTTAATTAATATGCCGAACGC
AATACTCGTAACAGGCAGACGACGAGCCTCCAAAAAGTATAAATAAATGGATCCTCTAGCTAGAGCTTTGCGTACTCGCA
AATTATTAAAAATAAAACTTTAAAAATAATTTCGTCTAATTAATATTATGAGTTAATTCAAACCCCACGGACATGCTAAG
GAAGGGCGAATTCGCGGNCCGTTAATTCAATTCCGCCCTATAGGTGAGTCGTATTACAATTCACTGGCCGTCGTTTACAC
GTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCN

P030 5'end with kp53 primer:

GNNNNNNNNNGNNGNNGNCNNNTTGCTTCTGGACCTTCNTTATTTCATCATGGGCTGGCGCTCAGCTGTTCGAATTGGCG
GCAGAGCGCCAGTGCTGCGCTACCTTTCGAACCGCTTACAACGCGTCGCCGCAACGCGGACGCTCTAAAAAGTAAAAAAA
AAGTGTAAAACAGTAATACGCAGTGCAACGCCACAGCAGCCGGAAACCAGGCAAAGCGCCATTCGCCATTCAGGCTGCGC
AACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATT
AAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGGCTGCTGCTCTAAACGAC
GCATTTCGTACTCCAAAGTACGAATTTTTTCCCTCAAGCTCTTATTTTCATTAAACAATGAACAGGACCTAACGCACAGT
ANCTGCTCTGCNGAGNGGNGGANGTTTAAAATTTNCTTCATGNGTGATGCCAANATTTGGNATATNNNNTGNCTCAGTAT
ATNAGCNTTATCAANGAAANATNTTCNNNTTAANNTTGAGGNGNGCCGGNCATNCCCGG

P030 3'end with Pyr2 primer:

GNNNNNNNNNGNNGGNNTNCGNGCCNTANNATAANCTATCCNTCANGANTTNGAGAGCNCGAAGANTGCCGANCGACGCA
GCTCCGCTACCACTACCACCGCAATGGCGCAGCAGTCGCGGTCGCTGTGCGCTGCTGTGCTCTGCTGTAAGCGCTCTTAC
ATTCCGCGCCCGAATTTTTCGTGCTTTTCCCTTGTTTTCCCTGTGGGCCAGCGNTTTGCTGCTGCTCGGACTCGGTTTGG
GCCAACACTTGTCGCCTCTTGGCCATTATGCAACGACAGCGAAACGAAGGTGCTCACNAAGTGGCCAAGCTGCTCTCTGA
TTGGTCGGCGGTCAGTGCCACGAGGCCAGCCGAAGTTCTCTCGCGAGAACCCAAGAGCACTCTCTCCCTCTCTTTTGGGA
GAGATGAGATAGCAAAGAAAAGCCAAAACACTAGAACAATCCAGAAGCCAAGTTTTCCGGCTTGCACATACGCCGTATAA
TCCGCATTTTTTGCGAACTCTAAACTCGAGTCTATCAGCTATAATATTACAATTCGTTCACTATAAACAAATNAACTAAT
ATAGTTTTGCTCAGAAATCGATGCACTATGATGAGAGAATAGTGAAAAACAATTCTCAGTTAATAGCTTTTAAACTGCGC
TGCAAATGGGAAAACAAATTANAATATACACATTNTCATGCCAAATAATAGGATATCCTATCCTTCATTATATAAGCATA
AACAATAAAAATATTCTAGCTTCGCTTTGATCCTCTTAGCTAGAGCTTTGCCGTACTCGCAAAATTATTAAAAAATAAAA
ACTTTAAAAATA

P0093 5'end with kp53 primer:

GAGGNAGNTTNNTNTATCCGTTCCCTTTGCATTTATATGGTAAGAGCGCTTACAGCAGAGCACAGCAGCGCACAGCGACC
GCGACTGAGCGCCATTGCGGTGGTAGTGGTAGCGGAGCTGCGTCGTTCGGCACTCTTCGGCTCTCTCTCTTCGGCTGGCG
CTCAGCTGTTCGAATTGGCGGCAGAGCGCCAGTGCTGCGCTACCTTTCGAACCGCTTACAACGCGTCGCCGCAACGCGGA
CGCTCTAAAAAGTAAAAAAAAAAGTGTAAAACAGTAATACGCAGTGCAACGCCACAGCAGCCGGATAAAAACAACAACAAA
AACTGCGCATGTAAAGCTGTGTTTATATTACTTGTTTTTTTTTTTACGGTGTCTCGCTGTTTTTGCTTTCGTCGCAGACA
ACAAAAGTGGCAAAGTTTTCGCCACGGCATTGCTCATACGCCCCGTCTCCTGCAAATTCGCTCACACTCACACGCGCGCC
AATACCAGCGCACATTCACATATATACAAGTGTACGCGCGCGTTTTTCAGTGTGTTTCGTTTCGTTTTTTTTTTCTGTTT
CTGTTGGTGACATGCAAAAAGCAACAAAAAAATAATAATAAATACATATAACGAAAACGATTAGTTCGGCGTTTTCTCGT
AAAAGTGGGCACCAAAATGCATCAGCCCAGGTGGAAATCAAAACAAAAACAAACCGAAAAATAAATAAATCATTTATTAT
TTAAACATCGCAAAGTAAGCTTCTNCTCGCACACAAGCACACACAAACTCGCGCGCTCATAGACTGGCTCACATACACAC
TAGCATACACATNTNACTCACACGCAGATGGAGCAACAAGGTTGCGTGCCACAAAAATTTCATTTCGCAANTTTTTGGCG
AATCTGGTNGGNTTTAATTTC

P0093 3'end with Pyr2 primer:

GAGTTTAANCCATTGGACTCTCCTTCCGCGCCCGAATTTTTCGTGCTTTTCCCTTGTTTTCCCTGTGGGCCAGCGTTTTG
CTGCTGCTCGGACTCGGTTTGGGCCAACACTTGTCGCCTCTTGGCCATTATGCAACGACAGCGAAACGAAGGTGCTCACG
AAGTGGCCAAGCTGCTCTCTGATTGGTCGGCGGTCAGTGCCACGAGGCCAGCCGAAGTTCTCTCGCGAGAACCCAAGAGC
ACTCTCTCCCTCTCTTTTGGGAGAGATGAGATAGCAAAGAAAAGCCAAAACACTAGAACAATCCAGAAGCCAAGTTTTCC
GGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGC
GCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTGTTGG
GAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGT
AACGCCAGGGTTTTCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGA
GCTCACCGCGGTGGCGGCCGATAGGCCACTATGGATCTGGATCCTNTACTAGAGCTTTGCGTCTCCAAATATTAAAATAA
AACTTTAAAATAATTCTCTAATAATATATAGTTAATCAACCCCNCGCATCTANGANGCGATTCNTAACCTCNGACTATCC
TTATGAGGTAATTTNCTTGNCGATATGGCTACTGTTCTNGGGAATGTTCCCTACAATCNCCACNTCACCGGAGCTAAGNN
AAACCTGGGGGCTATANTACTACTNCTTATTGN

P0103 5'end with kp53 primer:

GNNNNNNGNNNGNGGNTTNCNATCCTTNNCCTTNCGTTATTTATCATGGGCCAGCGTTTCGCTGCTGCTCGGACTCGGTT
TGGGCCAACACTTGTCGCCTCTTGGCCATTATGCAACGACAGCGAAACGAAGGTGCTCACGAAGTGGCCAAGCTGCTCTC
TGATTGGTCGGCGGTCAGTGCCACGAGGCCAGCCGAAGTTCTCTCGCGAGAACCCAAGAGCACTCTCTCCCTCTCTTTTG
GGAGAGATGAGATAGCAAAGAAAAGCCAAAACACTAGAACAATCCAGAAGCCAAGTTTTCCGGCTTGCACATACGCCGTA

TAATCCGCATTTTTTGCGAACTCTAAACTCGAGTCTATCAGCTATAATATTACAATTCGTTCACTATAAACAAATAAACT
AATATAATTTTGCTCAGAAATCGATGCACTATGATGAGAGAATAGTGAAAAACAATTCTCAGTTAATAGCTTTTAAACTG
CTCTGCAAATGGGAAAACAAATTAAAATATACACATTTTCATGCCAAATAATAGGATATCCTATCCTTCATTATATAAGC
ATAAACAATGAAAATATTCTAGCTCGCTTTGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGT
GCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTCTG
CTGCTCTAAACGACGCATTTCGTACTCCAAAGTCGAATTTTTTNCCTCAAGCTCTTATTTCATTAACAATGAACAGGACC
TACGCACAGTAAGGGCGAATTCGTTTAAACCT

P0103 3'end with Pyr2 primer:

GNNNNNNGNNNGGNGGGTNNCNNTCCTTGATGCGCCGGCCACAGGGAAAACAAGGGAAAAGCACGAAAAATTCGGGCGCG
GAATGTAAGAGCGCTTACAGCAGAGCACAGCAGCGCACAGCGACCGCGACTGAGCGCCATTGCGGTGGTAGTGGTAGCGG
AGCTGCGTCGTTCGGCACTCTTCGGCTCTCTCTCTTCGGCTGGCGCTCAGCTGTTCGAATTGGCGGCAGAGCGCCAGTGC
TGCGCTACCTTTCGAACCGCTTACAACGCGTCGCCGCAACGCGGACGCTCTAAAAAGTAAAAAAAAAGTGTAAAACAGTA
ATACGCAGTGCGACGCCACAGCAGCCGGATAAAAACAACAACAAAAACTGCGCATGTAAAGCTGTGTTTATATTGCTTGT
TTTTTTTTTTACGGTGTCTCGCTGTTTTTGCTTTCGTCGCAGACAACAAAAGTGGCAAAGTTTTCGCCACGGCATTGCTC
ATACGCCCCGTCTCTTGCAAATTCGCTCACACTCACACGCGCGCCAATACCAGCGCACATTCACATATATACAAGTGTAC
GCGCGCGTTTTTCAGTGTGTTTCGTTTCGTTTTTTTTTCTGTTTCTGTTGGTGACATGCAAAAAGCAACAAAAAAATAAT
AATAAATACATATAACGAAAACGATTAGTTCGGCGTTTTCTCGTAAAAGTGGGGCACCAAAATGCATCANCCCAGGTGGA
AATCAAAACAAAAACCAACCGAAAAATAAATTAAATCATTTATTATTTAAACATCGNAAAAGTAAGCTTNTNCTNCACAC
AAACACACACAAACTCGNGCGCTTATAGACTGGCTTACATN

P0110 3'end with Pyr2 primer:

GGGGGTGTTTTANNAGNGATCTCTTTGAAAGGCCCACACTNGTTTTGNCTAGGCCTCCNTGCAACGACAGCGAAACGAAG
GTGCTCACGAAGTGGCCAAGCTGCGCTCTGATTGGTCGGCGGTCAGTGCCACGAGGCCAGCCGAAGTTCTCTCGCGAGAA
CCCAAGAGCACTCTCTCCCTCTCTTTTGGGAGAGATGAGATAGCAAAGAAAAGCCAAAACACTAGAACAATCCAGAAGCC
AAGTTTTCCGGCTTGCACATACGCCGTATAATCCGCATTTTTTGCGAACTCTAAACTCGAGTCTATCAGCTATAATATTA
CAATTCGTTCACTATAAACAAATAAACTAATATAATTTTGCTCAGAAATCGATGCACTATGATGAGAGAATAGTGAAAAA
CAATTCTCAGTTAATAGCTTTTAAACTGCTCTGCAAATGGGAAAGCAAATTAAAATATACACATTTTCATGCCAAATAAT
AGGATATCCTATCCTTCATTATATAAGCATAAACAATGAAAATATTCTAGCTCGCTTTGATCCGTCGAGCGTATAACCAT
CTGTACAAAAAAGGATTTCCTTTGCCCAGTCGTACGACTTTGTCAGATGGTTATCAGATGTGGACATAAAAAGAGGATGT
TTGGATGTGGTCATAGACCTAATGGACAGTGATGGAGTTGATGACGCCGACAAGCTTTGCGTACTCGCAAATTATTAAAA
ATAAAACTTTAAAAATAATTTCGTCTAATTAATATTATGAGTTAATTCAAACCCCACGGACATGCTAAGGAAGGGCGAAT
TCCNCGGCCG

P0197 5'end with kp53 primer:

GNNNNNNNNAGNGGNNTNNNTTTGAANCCCGTCCCCTTATNTTATTTCATCATGATCAGTGGTTCTCAGTCCGTCGTTGA
CTTTCGAAGTGTGCAGTTCCCGAGAGCGGTAGAAGCAAGTAGTGGCAATCAATTGAAGACTAATCTTCCGAAGAAATATG
CGTGTTTTGGGGTCTAAGAAACATTTTGAGGCCAATCGACGTTGTGCTTCGTAACCTTTTTGTGGAAACTACTATTAAAA
TAGTTACTTAAATTATGAACCTACATATGTAGCTGTGCGTGGGTGTGTTTTTGTGTGTTCGGTGAAAAACGCAAATAC
AGAGTGTCAAAAGTAAACAAATTTGGTGCTGAACTTCTGGAGGTTGCAGCATAGGAGTCTTCAGTCTACAAAAGGTATCC
GACTTATTTGAATCCTAGCCGTTTTACCTCTTTTTCCCTCGGTGCTTCGTATATTCTGTTTTTCACATGCCTTTTTGTTT
TCGTCGTCTGCTTCTGTTGCGGCCAGAAAGAGAAGAGAAAACTTAATACATAAGCACATATGTGGATTGGGGCAAGCAGA
CGTCGTTGTTGTTTCCCCTGCTCTTGTATTATTTATATTTTTCTTTGGTTATAATTGTCTGCGTGGTTCTTATTGCTGCT
CTTTGTTTAGTTGCCGTTTCTTCTTCCACTTATATTCNTTTGCAAGTATATTGGTGTGTGCTNGCGGCTCTCTTTCTTTG
GGGTTCTTTCTCTCTATCTCTTTATTGGACTCTCTTCTCCTTCCCTATGTGTGTTTGNAGGCCTCGGCAGAAGACTGGCG
GATGTCCGGTTTAAGCCCNTNGATCGANGAATCCTTANGAGAGAACCGCCCCCAAGAACCCTT

P0197 3'end with Pyr2 primer:

GNGGGTTTAATAGTGATCTCTTTGAAGCCCCGANNTCTTTCTCGCTGCTGACCCTTTGGCTCGCTCTCAACGCAGTTTTG
GCGTCGCTGCAGTCCTCGCTTTTGTATGAGGGGTGGAGTAGTGGGGGTGTAGTTGCATCCTTGTTGAATTCAACAAAAAA
TAGTCTAAGTAAGCTAAGCTTACTGACAAATAAAGGTAGCCGTAATTTTCACTAATAAAAACACCTGTGATATTATTGGA
ATGGAAAGTTTTATGCTTTTAACGTAAATACAAAATTCCAAAGTATTTTACTCTAATTTCTTATTAGTATTTCTTTTTTT
AACGGAATAATATATAGATTATATTTTGAATGAATTGTATAGTAGGTACATATTAATGTGTAATATGTTCATATGTCACA
AAAAATATACATATGTTTAATTTGGAAAAATATCAACTTAGCTAGTTGATGCACTGCTTGGATTAGGTGTCGATAACAAT
GTGCACAAATAATATTCGAGTTTGACTTTCTACTTCTTTGAACTGTTACACTTGCACGTCAAGCGGTCCTGTGGCGCAAT
GGATAACGCGTCTGACTACGGATCAGAAGATTCCAGGTTCGACTCCTGGCAGGATCGAATTTTTTGGCGTATTTTAATTT
TTATTATCCCCAATATGTGGATACTACTCTAGTGTCATTCTTTGACATTCTATTTTTCAAGATATCCTTGACAATAATGT
TAAAAATACTAAAAGTGAAATTAAAAATTGTCATNCCAAAGCTTTAATGGNGGCTTTCTGGTTTACTTGGTTATAGCGTT
CA

P706 5'end with kp53 primer:

GGGGGGNNNNNGGGGTTTTNNNTAAGTGAANCCCTTTGNACTTACCCATTTCATCNTGGAACAGGGGCATGGTTGTTTGT
TTTTTGGGGGCATCAAGGGCATATCGGGCAGACATATTGACATCTGTGGAAAAATATATAAATTAATTGGGAAACTCAGG
GAAATTTCATTTTAAGATTGGAAATAAATTCAATTTTTAGACCACTGCCTTTTGATTCACTATATAATGATAATATAATA
TATAATAACTTTTCCAAACTAGCTCTGAAAAACCTTAAAATATTTGATCAAGTTATACATTTAAACACCGAAAATCATCA
TTCGCTCAGTTTACAAAAGCTATTAAATCACGTGATGTAATGATTAAGCATGGGTATTTCGCCCGTCGCAACCTTTTGAG
CTAACAACTTTTTTGTGAATGAAAATTGTAATGCAGTGGAGTAGATAAATGGATGAGGGATTGGGGCGTTGGGGCCTAAA
AACATGAAGCTGGAGGGGTGTTGAAGAACCCGTGAGGCGCAGTTTATTGACTCGCCATGTAAACAGTGTTGTGAGCCCTA
TTTTTAGAGTAGAACATCAAAACCGTCTATGACCAATTATGCAGATTTCTTGTTTTCTGGCACACACATCTGGCGGACAC
ATTTGCCCAACTGCAGTACTGATTTTCCCATTTCATTCTTTTATTTTTCTGGGTGTTTTTCCACCAGGAGTCGGCGAAAG
ATCCGGCCCCGGGAAACCAGGCAAAGCGCCATTCGCCATTTNAAGGGTGG

P706 3'end with Pyr2 primer:

GAGTTTAANCCNTTTNCCTGCCTAAGCAGCTGACAGACAGAGACGGCACGAGGCGGTCGGAGGGAGACAGGGCGAGACCC
CGCAAAAGGGCGAGGACTATGAGACTTTGGCTGCGGCACGAGCTGTGTCAAAGTTCGTTGACTGCATTCTGTGGTCGGTC
GTAGGGGGGGGGGGGGGAGAGAGGGGTTGGGATTGGGCTGCTGTCTAGTCGGCAGCGTCATAATACACTCGAAAAATGCAC

GGGCAGACCTCGGACGAGCGAAATCTGACAGAAAGTCACGAACGCAGCCCTCAACATGTCATTAACAGCAGGGCGCAGAA
ATAAAGCGGCCCGATTTACGGGGAAATAATAAAAGAGCCAAGCTGCAGCAGCAATTTCCTGGCAATTCGCGTACAAAGTA
AGTAAGTAAGTAAGTGTGCGTGGGCACCTCAAATGAAAATGAAAAATGGAAAAACGAAAAACAAGTGTTTCGGAATTCGA
AAGCATGCAATTGCAATTGCCGCCCCGACCGACTATCGATAACTGGCGGGGCGGCCACCTGTTCGATTGGAGCCTCGCTG
CCCGGAGTACTGGACGTAGGCACGGTGACATCTTCGGTGCCGGTAAGCGGAANGGTCNGAACANGANAGCGCCNANGGAC
CTTCCANGGNGAAACNCCTGTATCTTTATATCCTGACNCTTTTCGCCNCCTTNACTTNANCGTCNNTTTTTGNGAACTTC
NNAANGGGGCNTANCTATGAAAAACN

P898 5'end with kp53 primer:

GGGGGGGGNNNAGGGGTTTTTANANGCTTATCTTTCCGGAAANACCCCATNNNATTTTGCGGCNNACCAACCGGTTTCGC
TCTCTCGGTTTTATGGAGCATTTGCTTGGAAAGTCGCATGCGATAAGTGGCAGAGAGGATAGAATTTAAAAGGCCCCGTT
GTTGTTGCTGCTGCAGCACCAACAGCTGTTTTTTGTTTATTTCAATAAATTCTGTAGCATTGTCTGATAAGCACGTGGAT
TTTTTTCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGT
AACGCCAGGGTTTCCCCAGCCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTCTGCTGCTCTAAACGACGCATTTCGT
ACTCCAAAGTACGAATTTTTTCCCTCAAGCTCTTATTTTCATTAAACAATGAACAGGACCTAACGCACAGTAAGGGCGAA
TTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCT
GTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGGCCTAATGA
GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATG
AATCGGCCAACGCGCGGGGAGAGGCGGGTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGG
TCGTTCGGCTGGGGGGAAGCGGNATCAGCTCACTCAAAGGCGGNAAATACGGGTATCCACAGAATCAGGGGGANACCG

P898 3'end with Pyr2 primer:

GAGGNAGTTTNNTCTANNCCNTTTGGNCCCNGTTATTGTTGAATGTTAACCGATTGGCATCGGACAAAACGCACTGTTAG
CCGGCCTCGCACGTGTAACTTCCCATATGAGTCGCGCTGTTAACCGCTAAGGTTTAAAGTTTTCGCTGTTAATTCCAGCC
GAAGGAAATTCGAATGTAAGGAAAGTGGCTCGTTCGTTTGTTCGCTAAACGCAGGCGGTTAGTTTGAAAATTTTCTTCGA
CGAAGGCGGTTCTCACGCGGCTGTACACGCGGATGCAGATGCTCTGCTATCGGCGCTCATCGAGATACAAATTTACGTAA
AGTTCGCTGGCCAATTGGAAGTGCGCGTGCTGGAAAGTTGGGAGTTTTGGGCCATAAAACACAATTAAAAGTGCTAACGA
GAAGGAAACAAAGTTACCGAAACAAATTCAAACAGAAAACGCAAAGAAAACAAGCTACTTTGGCTTTAAAGATTTTAATG
GCCTATTGTGTGCGTGTAATTGCCTTGATTGATAACCGTTGACAGCGACTTTTGCACCCCGTCCCCGCCCGAAAAATTCG
AGAAATTATATTAAAAGCTGTCGGCAAGTGTGACCAAAGTGCGTTAGAATTCAATAAGTGAAGAAGCAGAAGCCCGAATA
AGACGGCCCTCAACCCCGCTTTTTACGCTTTGTTAGTGACGAAGTGCGAGTTGAGGAATTTTCAATTAAAATTACACACA
GAATCAATGCAATTATAGAGACGATTGCAAAGTGCGCACTCGATGCAATAAAAGCAATCATTTAAATGGATTAATTGACC
TGAAAGAAATTCCTGGACATAATTCAAAGTTCCTGCAAAGAAGTTGGAAATTCTGNATTTGCAAGTGCTTATCTACGAAA
ANGNGAGTTGGTGGAAAAA

P0937 5'end with kp53 primer:

GGGAGTTTNNTCNNNTCNCTTTGANCTTCCTTATTTATTATGGTCGCAACGTTACCTTACCTGTGCGCTTCGCTTTTGTT
TTCTCTTCGTTTTCTCGTCTCGTCTTCGCCGCCGAAACATGTGGGTTCCGTTTTCGATTTGCTTCAATTAAAAAAGAAAC
GAATATTTCCAAAACGTCGCGAAGTTACGCGCAAGAAGTGCAGCAAAAGCAAATGTACTGAAAGCGAGGAATACATTCTT
CTTCAAAAAAAAATACCATCAAAAATAAAGTAAGAAAAAGTTTTTTCTGCAAGAGAGCTTAAGTAAAAAAAGAGCAAGGA
TGCCGCCAAAATAACAGGAGTTAATCCAAAATAAATACTAAAATACCAAAACCCCAAAAAATACCAAACTAGCAGTGACA
GAGAAAAGCAAAAGTTAAGCACCAAAAAAACTGAGCAAAGAAAGTAAGTTGATGATCGGTTTTGCATGAATTCCGCGCAC
TTAAGGCGGACAATCAGACTTTTTAGTGGATTAGTTTTCGGTTTTTGCTAGATTCTGGGTGGGTTATGTATTAAGAGTTG
AAGTAAGGTTAAATCTAGCCCCTAGACCGACCCCTCCCTTTGAACACTTGCTCGATGGATGGTCACCGGAAACCAGGCAA
AGCGCCATTCGCCATTCAANGGGTGNCAA

P2103 5'end with kp53 primer:

NNGGNNGNNTNNNTTTAANCCNNTCCCTTTGNATTTATCATGGTCCTGACGAAGATCGCAAGAAGAGGGTTCGTAACTTA
CACGAACATGTTACTAGACAGGCCCCGAGTCGCCTGCTGACGGGGCCCGAAAACACGAGAGCCGAGCGCTGGTGAGTCCT
GACGAAGAGCGCAAGAAGAGGGTTCTTAACTTATATATAATAAAAAGTAAAAAAAAAGTGCATTGAAATCAGAGTCAGAA
CATTGGCACGGAATTTTTAATATCATTTTTTCCACCAAATGTACTCGAACTTTGGATTCAGCCGCGTTGCACATCTGTAT
GTTTATTTTCCAATTTTATTTTCACTTACTGAATTACTTGGCGTCGTTTGTTTTTGCCTCCTAGCTTATTTACTTAGAAT
TTTATGGCCAAACTGATTTATTTGGCAAAATATTAGCAAATAACAGCAAGGTGAAGGTTAGCATGGAAAGTCCCCCGGAA
ACCAGGCAAAGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGC
CAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAAC
GACGGCCAGTGAATTCGGCTGCTGTCTAAACGACGCATTTCGTACTCCAAAGTACGAATTTTTTCCTCAAGCTCTTATTT
TCATTAAACAATGAACAGGACCTAACGCACAGTAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGG
TTAATCTTGAGCCTTGCGTAATCATGGNCATAGCTGGTTNCTGNGTGNAATGGTATTCCGTCACAATTCCACACACATAC
GAGCCGGAGCATTAN

P2103 3'end with Pyr2 primer:

GAGTTTAANCCCATTGGNCAGGCCTCACCAGCGCTCGGCTCTCGTGTTTTCGGGCCCCGTCAGCAGGCGACTCGGGGCCT
GTCTAGGAACATGTTTGTGTATGTGTGCATTCGGAACAAGTGCCGTTGGTCGCACTCAGGGTGAGGGGTCAACGGGGGAA
GCGGATATAAAAGCAGCGGGGCGGGAGAAGAGCCCCAGTCTCGAACGGACACATAACGGAACCGCTAGCAGATCTGCGCC
ACATCGCGTCCGAAGATAACGAAGCTCTATGGGAGATTAAACATAGTTAAAGCTTTGCGTACTCGCAAATTATTAAAAAT
AAAACTTTAAAAATAATTTCGTCTAATTAATATTATGAGTTAATTCAAACCCCACGGACATGCTAAGGAAGGGCGAATTC
GTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTG
TGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGT
GAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTNCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAA
TCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCTCGTTACTGACTCGCTGCGCTCGGCGTT
CGGNTGCGCGAGCGGTATCACTCACTCAAAGGCGGAATACNGGTATTCACAGAATCAGGGGGATACGCAGGAAAAACATG
TGAGCAAAAGGCNCAAAAGGCCCGGAACCTAAAAAGGCCGCTTGTTGNNNTTTTCCTAAGCTCCGCCCCCTACAGCTTNC
AAAAATNACCTNAAGTAAAGGGGNAAACCCCCNGGCTTNAANAACCGCCGTTTCCCCTGAA

P2356 5'end with kp53 primer:

GGGAGTTTNNTCNNNTCCTTTGACCTTCNTTATTTCATCATGCATATGTTGTTAAATGTATGAACGAAACACACACAGAC
GAACTCCGTGCACGTAACGAACATTAAGCGCCTTATTTTAGTTTGATTTTGATTTGATTTTTATTTGAATTTTTCGCCGC
TTCGTCGTTATCCTCCCGTCTGTATGCGTGTTGCATGTTGCTATTGCTATTGCGTATGTGAAATCGCAACTGCCTCCTAC
GAACAACCATGACAACAACTACAACATCTACAACAACGTTGGTCGGACTGGAAACTCCAATTGGGATGGCTAACGAAAAC
AACAAAAACAACAACAACGACCAGACGTTGCAACGCGCCCGCCTTGCCTTTTCCAGTGGAAAGACCAGGAATGTCAGCTT
TCGGTGAGTTTACTTTTATTTCGGCACTTATTCGCTTGCTGTCAGTTAATTTTTTGCAAAGGCTCCTCGTCTTGGCCAAC
ATATTGCGTTAAAACTGCCGCTTGAGTGGAGATTGTGTCATATATTTCGCTGATTACATTGGTTGGGAGGATCTGGAAAG
TGGAAGTGGGTTAACTTCCAGCAAGCGGGACTGGAATTAAAATCGCCGTGGAAGTTATTTGAAATCTTCACATCTTCATT
CTATTCCGCTTTTGTTTTGAATTTCATTGATTTGTGATTTAATTTTTTTTTTAATTTATTTAACTCGATGCGGAGTTTTG
NATAATTTGACATTTATCTCTTCTTTGGCTTTAGATGACTTCTTTTAATCGCATAGTTTGATTTAATATCCCTT

P2356 3'end with Pyr2 primer:

GGNNGGTNNANNAGATCCCTTTTGAAGAACCCAGNGTCATTTAAAGTTAATAATCGTTACACATCTCTTTCTCTCGCTCG
CAATAAATTGCCTAATTGGACACTCACATCGTCAAAATTGGCCATTCTGTCGGACTCGGATTCGGATTGGAGCTGCTCGG
GTTGTTGTTGTTGACTTTCCGGCGGGAATGTGCGGATTAGAGGGCCTGGCTATCTGGCAGACACAAATGCCCATGACGAT
CCCAGCTCACCCACATCCGAGTAGCACTGCTCCCATGCGCGATTGGCAAGTGCACCATCTTGATCGGGCGATAAGGGGCC
ACCAGCAAACAACGAGGCTTCACATGGGGGTCAATTGTTATCACTATATGCACAGCAAATACTATATACATTCGACACAT
TCAATTGAGCAAGAAGGCAAATTCCATCGAATGCCAGTTAAACGCAATTAGGGCAATAAAACAATAAAGGGAATCGATAT
AGTAGGAGTGGGCCGACTTTAAGGAAAGACACGCAAGGCATCCGAGTATCTTTAAGTACCTTCTTTCCATTCACGAAAAA
CACCAAAACCGAGTTAGACATCGTGAAGTGCTATGTATCTGTAGCTCACGGTATCTCGCTGTATCTCAGTACCAATGCCT
ATATAAGTGGTTAGCCTGCAATTCCGAAGCTCGTTTACATATATGGAGGGGAGGAGGTGCGTTCCTTGAATGTAAAGCGA
AAACAAGTTTTTTATTTCAAAAATTAGAAAACCCGACAACCCTTCNTGTNCCCCGCCGATTAAAAAAACANCCAACCGNT
TTNGNGCCG

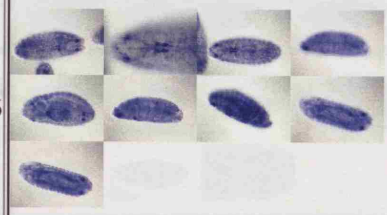
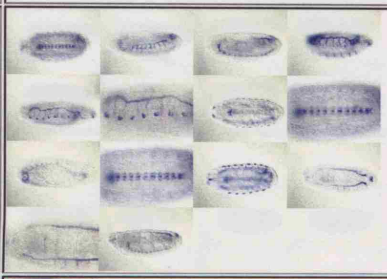
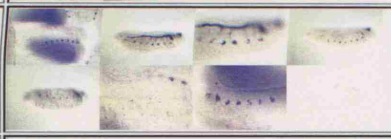
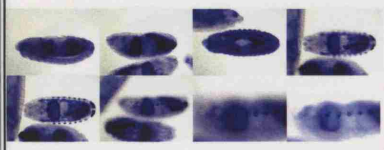
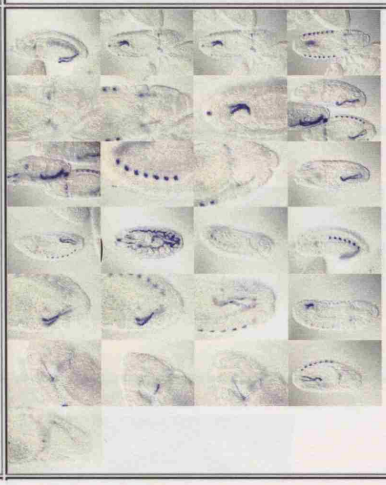
Appendix 3. Embryonic Expression Profile and *In Situ* Expression Pattern of Genes Expressed in Oenocytes

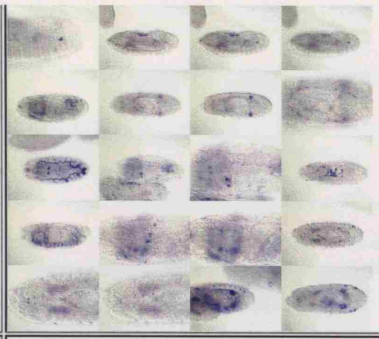
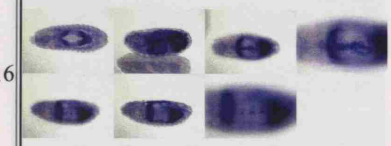
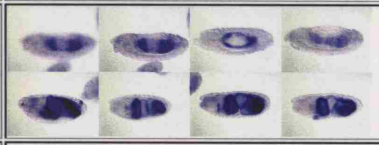
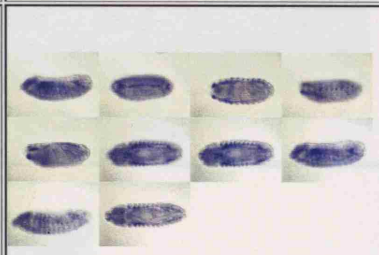
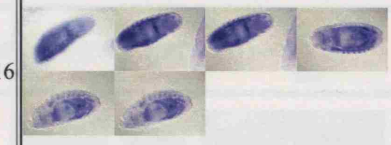
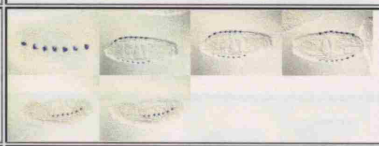
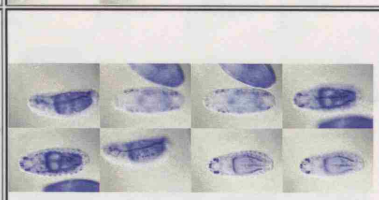
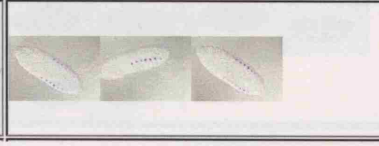
The following four pages show the results of a search performed for genes expressed in oenocytes using the BDGP embryo *in situ* database (See Materials and Methods). All 21 genes found are discussed in Section 4. In addition, GH06606, a transposable element was also found. Of particular interest is the expression of 4 genes exclusively expressed in oenocytes (*CG14615, CG18609, Cyp4g1* and *CG17562*) and 3 more with a very restricted expression pattern (*CG6921, CG11567, CG31095*).

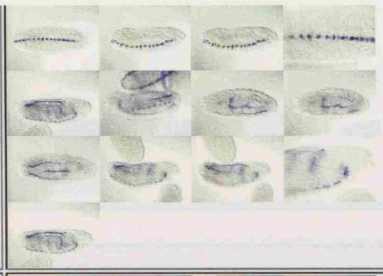
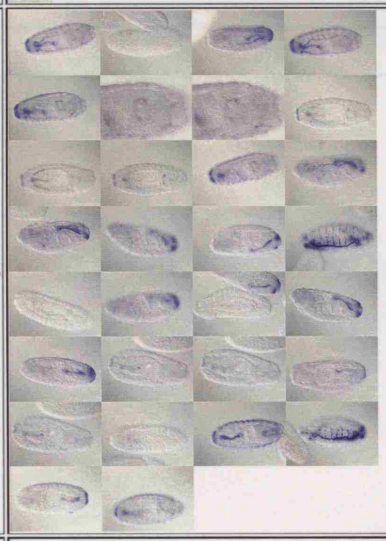
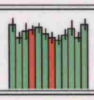
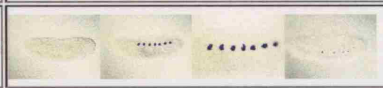
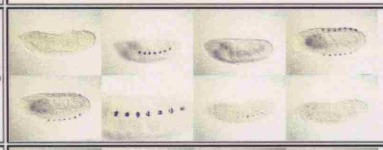
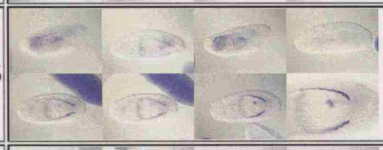
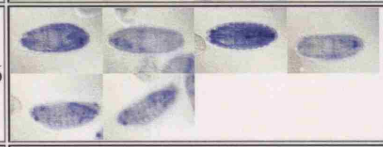
The first column of the figure shows the name of each gene, together with the cDNA clone used for *in situ* hybridisations [square brackets], the gene cytological location and in some cases the identity of the gene product. An independent microarray expression profile is shown as means of validating the *in situ* results. Images of embryos are not always oriented anterior to the left, dorsal to the top.

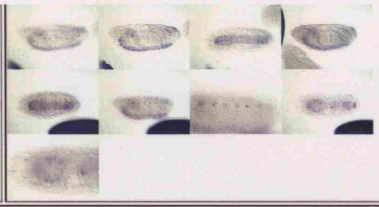
BDGP Gene Expression Query Results

| Search | | Advanced | | Batch | | Summary | | Home | | ImaGO | | Help |

Gene	Array Profile	Stage	Image	Body Part
Atet (CG2969) [RE01860] **Map**: 24E1-24E3 **Function**: ATP-binding cassette (ABC) transporter		stage13-16		lymph gland need new term(sensory system head) embryonic dorsal epidermis embryonic/larval dorsal vessel embryonic ventral epidermis amnioserosa embryonic/larval posterior spiracle ***embryonic/larval oenocyte*** embryonic/larval circulatory system
CG31764 [RE18101] **Map**: 33E3-33E4		stage13-16		ventral nerve cord ventral midline embryonic central brain embryonic/larval posterior spiracle ventral midline glia ***embryonic/larval oenocyte*** embryonic/larval tracheal system ventral midline neuron
CG14615 *[RE68036]* **Map**: 20B1-20B1 **New Release**		stage13-16		***embryonic/larval oenocyte***
Cat (CG6871) [RE33242] **Map**: 75E1-75E1 **Function**: catalase calcium-dependent cell adhesion molecule heme binding **New Release**		stage13-16		embryonic/larval fat body embryonic midgut crystal cell embryonic hindgut embryonic anal pad embryonic midgut chamber ***embryonic/larval oenocyte***
CG6921 [LD14839] **Map**: 94B4-94B4		stage13-16		embryonic large intestine embryonic hindgut ***embryonic/larval oenocyte***

CG31361 [RE56367] **Map**: 87A8-87A9 **New Release**		stage13-16		ventral nerve cord lymph gland gonadal sheath ***embryonic/larval oenocyte*** embryonic salivary gland gonad
CG11151 [RE42326] **Map**: 12B7-12B7 **Function**: estradiol 17 beta-dehydrogenase **New Release**		stage13-16		embryonic midgut embryonic/larval dorsal vessel embryonic midgut chamber ***embryonic/larval oenocyte***
CG9527 [RE34879] **Map**: 26D3-26D4 **Function**: pristanoyl-CoA oxidase **New Release**		stage13-16		embryonic midgut ***embryonic/larval oenocyte***
NHE2 (CG9256) [RE21674] **Map**: 39A2-39A3 **Function**: sodium:hydrogen antiporter		stage13-16		embryonic/larval muscle system macrophage embryonic foregut crystal cell gonadal sheath embryonic dorsal epidermis embryonic/larval dorsal vessel ***embryonic/larval oenocyte*** embryonic/larval hemocyte embryonic salivary gland gonad
CG7920 [GM14349] **Map**: 99D1-99D1 **Function**: 4-hydroxybutyrate CoA-transferase		stage13-16		embryonic foregut embryonic large intestine embryonic hindgut embryonic Malpighian tubule dorsal prothoracic pharyngeal muscle amnioserosa ***embryonic/larval oenocyte***
CG18609 [RE06553] **Map**: 55F1-55F1		stage13-16		***embryonic/larval oenocyte***
GH06606 [GH06606]		stage13-16		embryonic foregut sensory structure need new term(sensory system head) embryonic Malpighian tubule embryonic/larval pericardial cell embryonic/larval dorsal vessel ***embryonic/larval oenocyte*** embryonic maxillary sensory complex embryonic/larval circulatory system embryonic labial sensory complex
Cyp4g1 (CG3972) [GH05567] **Map**: 1B3-1B4 **Function**: cytochrome P450		stage13-16		***embryonic/larval oenocyte***

CG3132 [HL01076] **Map**: 87A4-87A4 **Function**: beta-galactosidase		stage13-16		ventral midline embryonic head epidermis embryonic/larval dorsal vessel embryonic optic lobe ***embryonic/larval oenocyte*** embryonic/larval circulatory system
CG3328 [GH24458] **Map**: 60C1-60C1		stage13-16		embryonic large intestine embryonic hindgut embryonic anal pad embryonic ventral epidermis ***embryonic/larval oenocyte*** embryonic/larval garland cell
Cpr (CG11567) [LD46590] **Map**: 26C3-26C3 **Function**: NADPH-ferrihemoprotein reductase		stage13-16		amnioserosa ***embryonic/larval oenocyte***
CG17562 [RE20520] **Map**: 89D5-89D5		stage13-16		***embryonic/larval oenocyte***
CG12262 [LD22634] **Map**: 66A10-66A10 **Function**: acyl-CoA dehydrogenase		stage13-16		embryonic/larval fat body crystal cell embryonic Malpighian tubule embryonic proventriculus amnioserosa ***embryonic/larval oenocyte***
CG31095 [LD21010] **Map**: 96F2-96F2 **New Release**		stage13-16		ventral nerve cord embryonic central nervous system ***embryonic/larval oenocyte***
CG12428 [SD09912] **Map**: 98B2-98B2 **Function**: carnitine O-octanoyltransferase		stage13-16		embryonic hindgut ***embryonic/larval oenocyte***
nesprin (CG31649) [GH18470] **Map**: 25C9-25C10		stage13-16		embryonic dorsal epidermis embryonic ventral epidermis amnioserosa embryonic/larval posterior spiracle ***embryonic/larval oenocyte***

CG31092 [LD11117] **Map**: 96E10-96F1 **New Release**		stage13-16		ventral nerve cord embryonic central nervous system embryonic central brain neuron ***embryonic/larval oenocyte***

Showing items 1 to 22 of 22.

all | 1:22

Format with Image Array Profile [?] Redraw

| Search | | Advanced | | Batch | | Summary | | Home | | ImaGO | | Help |